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TICK-TRANSMITTED PROTOZOA IN THE PERIPHERAL BLOOD OF CLINICALLY HEALTHY CATTLE IN PALESTINE

By

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INTRODUCTION

It has been known for many years that four tick-transmitted protozoan parasites commonly cause disease among cattle in Palestine. These parasites are (*a*) *Anaplasma marginale*, (*b*) *Theileria annulata*, (*c*) *Babesia bigemina*, (*d*) *Babesiella berbera* (Sergent *et al.*, 1924). Figures 1 to 4 show these parasites in blood smears from animals clinically affected. In Palestine the parasites have been collectively described as the causal agents of "tick-fever," a convenient term that will, for the sake of brevity, be used in this paper when reference is made to them as a group.

So common is "tick-fever" in Palestine, that whenever a bovine shows a rise of temperature, or other evidence of disease, the first suspicion is that it may be affected with "tick-fever." If the mere presence of one or other parasite were diagnostic of disease the matter would be simple. This, however, is not the case. It is recognised that carriers of "tick-fever" parasites exist, and in cases where these parasites are few in number and in which the blood smear shows little abnormality, the question inevitably arises, "Is this, or is this not, a carrier animal?"

The object of this paper is to provide some sort of answer to this question, and to indicate to the clinician the limitations of a laboratory examination of a single blood smear. It is also to make clear that in a number of cases an adequate reply cannot be given in cases where a single blood smear is examined either to incriminate "tick-fever" if the clinical symptoms resemble "tick-fever," or to eliminate "tick-fever" if the clinical symptoms more strongly suggest some other disease.

Accepting the general principle that complete understanding of the apparently diseased is impossible until the apparently healthy is thoroughly understood, this paper deals with examination of blood smears made from clinically healthy animals. As far as possible, the conditions of examination have been kept the same as those obtaining in cases of suspected disease. That is to say, only a single smear, prepared under field conditions, from each animal has been examined, and the examination has been brief.

To the best of the writer's knowledge, no results of a systematic examination of blood smears from healthy cattle in Palestine have hitherto been published, although numerous veterinary surgeons who examine smears from their own cases of suspected "tick-

fever " have expressed verbally their opinions as to the percentage of carrier animals in the country. Adler and Ellenbogen (1935), however, have published a paper in which are included data concerning the examination of blood smears from 54 healthy but splenectomised native calves from the Jerusalem district. Their findings will be referred to later.

METHOD OF EXAMINATION

All smears were received in the laboratory unfixed, and were thereafter stained Giemsa. To obtain some basis for comparison, examination of each smear was timed to last five minutes. The criticism may be offered that a five-minute examination does not exhaust the possibilities in any blood smear. This, it is admitted, is true, but under normal working conditions in a busy laboratory it is seldom possible, unless for some special reason, to allot more than five minutes to the examination of a single slide.

Examination was carried out using a magnification of approximately × 1,000, and as an indication of the number of red corpuscles scrutinised in five minutes it may be stated that the speed of examination was about 12 to 15 fields per minute.

Notes were made on the presence or absence of parasites, their form, and approximate number, and also on the appearance of the red blood cells. The work was carried out during the period February-December, 1944, and, in all, 550 smears were examined from cattle of all ages and from districts representative of the different climatic areas (the humid coastal plain, the hills, and the hot Jordan valley). An effort was made to include cattle of all breeds—native, and native crossed with various imported breeds, especially Friesian. Examination of cattle for signs of clinical good health was carried out by Government veterinary officers, and in the case of the Government Farm by the farm manager.

Presentation of Data. Throughout the examination smears were classified according to the number of parasites found, thus : (*a*) Parasites rare, not more than three parasites found ; (*b*) parasites few, four to about ten parasites found ; (*c*) parasites in small number, about one parasite found per three fields. In no case were parasites more numerous than about one per three fields. It had been intended originally to include in the data presented figures indicating the number of parasites found. At the end of the examination, however, it was obvious that the important point was not whether two or 20 parasites were found, but whether or not they were found at all. For the sake of simplifying the tables, therefore, the number of parasites found has been omitted, and it is to be understood that in no case were they numerous.

Besides data concerning the examination of single smears from 550 different animals, results of repeated examination of smears from a few healthy animals are given with a view to assessing the value of the examination of a single smear. The animals used for the preparation of several smears over a period of weeks were housed in the laboratory stable, and examination of these smears was not timed to last five minutes ; the time spent on examination was variable, and the findings are treated separately.

Single Smears. Table I presents the findings for all smears from cattle of all ages and from all parts of the country. The term " native " is used to signify cattle uncrossed with imported breeds.

EXAMINATION OF SMEARS

TABLE I

Group	*No of animals*	*Anaplasma only*	*Theileria only*	*Anaplasma and Theileria*	*Babesia bigemina*	*Babesiella berbera*	*No parasites found*
Native cattle	200	30	37	14	0	0	119 (60%)
Other cattle	350	82	67	27	1	0	173 (49%)
Total	550	112	104	41	1	0	292 (53%)

It will be seen from the data in this table that parasites were found in approximately 47 per cent. of all single smears examined.

TABLE II

District	*No of animals*	*Anaplasma only*	*Theileria only*	*Anaplasma and Theileria*	*Babesia bigemina*	*Babesiella berbera*	*No parasites found*
Haifa ...	99	27	18	4	1	0	49 (50%)
Jaffa - Tel - Aviv ...	45	10	5	3	0	0	27 (60%)
Gaza ...	53	15	5	0	0	0	33 (62%)
Jerusalem	102	20	8	1	0	0	73 (72%)
Tiberias ...	251	40	68	33	0	0	110 (44%)

Table II presents the same data as in Table I in a different form, namely, according to the district from which the smears were obtained. The districts are representative of somewhat different climatic areas: (i) Haifa—the northern end of the coastal plain; (ii) Jaffa-Tel-Aviv—the centre of the coastal plain; (iii) Gaza—the southern end of the coastal plain; (iv) Jerusalem—the hills; (v) Tiberias—the Jordan valley. This division into districts has been made because there is a general impression among veterinary surgeons in Palestine that bovine carriers of "tick-fever" parasites are more numerous in the Tiberias area than elsewhere, and less numerous in the Jerusalem area than in the rest of the country. The figures indicate that this impression is apparently justified, but they cannot be interpreted too literally for the reason that cattle may be moved at will from one part of the country to another.

Table III summarises the findings of parasites according to age of the animals. The ages of 306 animals from which single smears were obtained were known to within about six months, and these animals only are included in the table. The particular age groups chosen were selected because it is generally believed that animals become carriers during calfhood, and continue to be carriers through-

TABLE III

Age Group	*No of animals*	*Anaplasma only*	*Theileria only*	*Anaplasma and Theileria*	*Babesia bigemina*	*Babesiella berbera*	*No parasites found*
2 years and under ...	81	24	15	5	0	0	37 (46%)
2½ to 6 years	164	37	32	14	1	0	80 (49%)
Over 6 years	61	8	14	7	0	0	32 (52%)

out their lifetime. The figures in this table appear to confirm this belief, and during the examination of the data for preparation of the table there was no evidence suggesting that parasites are found more often in smears from animals of any particular age.

Several Smears from the Same Animal. In the paragraph headed " Method of Examination," it has been admitted that five minutes is not a long time to spend on the examination of a single blood smear, and it was felt that a more prolonged examination, or the examination of another smear taken on another day, might reveal the presence of a greater number of carrier animals. That this suspicion was justified is made clear by the data included in Table IV. Indeed these figures are rather remarkable. For example, in the case of bull 85, *Theileria annulata* was found in only one smear out of 23 examined over a period of 24 weeks. In all these animals

TABLE IV

Animal	*Period of examination*	*Number of smears examined*	*Number of times parasites were found*			
			Anaplasma	*Theileria*	*Babesia*	*Babesiella*
	Weeks					
Bull 41/4 ...	64	58	0	24	0	0
Calf 54 ...	4	5	2	0	1	0
Bull 3 ...	16	17	0	4	0	0
Bull 4 ...	4	5	0	1	0	0
Bull 85 ...	24	23	0	1	0	0
Bull 5 ...	36	33	0	7	0	0
Calf 8818 ...	3	4	0	0	1	0

the parasites were rare, and the results indicate that in such cases they are not likely to be found in more than some 50 per cent. of single smears made at random. It is interesting, also, to note that *Babesia bigemina* was found in two different animals when repeated smears were made (cf. once out of 550 single smears).

The figures given in this table very largely falsify the conclusion that might be drawn from those in Table I, namely, that some 47 per cent. of cattle in Palestine are carrying " tick-fever " parasites. This figure must be greatly increased; it is almost certainly a minimum. It might be justifiable, indeed, to make such a general

statement as " repeated examinations of smears of peripheral blood would probably reveal that the great majority of cattle are carrying parasites."

FORMS OF PARASITES FOUND

Figures 1 to 4 illustrate the forms of " tick-fever " parasites seen in clinical cases in Palestine, and it is to be understood that for the sake of illustration numerous parasites have been depicted. In cases of Anaplasmosis and Theileriasis parasites are commonly as numerous in single, random smears as shown in the figures, in *Babesia bigemina* infection it is less common to find so many parasites per field, and in *Babesiella berbera* infection the finding of eight parasitised erythrocytes (as illustrated) in one field is definitely uncommon. However, the purpose of the illustrations is to indicate the form rather than the number of parasites.

In smears from clinically healthy cattle the Anaplasma parasite occurred as a single, marginal dot. In no case were two parasites seen in the same red cell. The Theileria parasites were almost all " signet-ring " forms, but 22 " rod " forms (15 different cattle) were found out of the total smears examined. As in the case of *Anaplasma marginale*, no red cell containing more than one parasite was found. *Babesia bigemina* parasites were found, in all, three times ; once in an animal from which one smear was examined, and twice in animals in which more than one smear was taken. In two of these cases only one parasitised red cell was found, containing a clear " double-pear " form ; in the third case two or three parasites were noted. *Babesiella berbera* parasites were not found in any smear from a clinically healthy animal.

EXAMINATION OF THE RED BLOOD CELLS

Anaemic changes in the blood, as determined by visual examination of stained erythrocytes, are commonly found in cases of Anaplasmosis and *Babesia bigemina* infection ; they are more rarely noted in cases of Theileriasis. A record, therefore, was made of any abnormality seen during the examination of smears from healthy cattle. Of the 550 single smears, 33 showed slight anisocytosis, and in six occasional punctate basophilic cells were found. No nucleated red cell (seen occasionally in clinical cases) was found. There was no apparent correlation between these slight anaemic changes and the presence of parasites.

DISCUSSION

The examination of blood smears from clinically healthy cattle in Palestine indicates that a high percentage of animals are carrying tick-transmitted parasites. *Anaplasma marginale* and *Theileria annulata* are very commonly found, but are never numerous, and *Babesia bigemina* parasites may be found occasionally. *Babesiella berbera* parasites were found in no case.

It is pertinent to compare these findings with other published data. Adler and Ellenbogen (1935) reported that in smears prepared from 54 splenectomised native calves from the Jerusalem area they found Anaplasma in eight, Theileria in one, *Babesia bigemina* in 17, Theileria and Babesia in one, and Babesiella in one. One marked difference in these findings from those here recorded is at once obvious—the figure for *Babesia bigemina*. The large number of splenectomised animals found to be carrying this parasite suggests that it is very scanty in the peripheral blood of normal, healthy animals and is, therefore, liable to escape microscopic detection. It is felt most certainly that since *Babesia bigemina* is known to be carried for years after infection (Wenyon, 1926), and since disease due to this parasite is common in Palestine, the finding of this parasite only once in 550 single smears does not represent the true measure of infection.

Tchernomoretz (1943) has summed up the position regarding *Babesiella berbera* infection as follows: " We have noticed fatal results in cases of infection with *Babesiella berbera* in which parasites were easily detected in blood smears, in others where they were scanty, and in a number of cases in which, as judged by microscopic examination, the blood had become negative." In the peripheral blood of clinically healthy animals they are apparently very rare, but that carriers of this parasite exist has been demonstrated by the work of Adler and Ellenbogen to which reference has already been made.

In conclusion, therefore, it must be stated that in certain circumstances the examination of a single blood smear is not an adequate method of diagnosing " tick-fever." If numerous parasites are found a diagnosis can, of course, be made. But—and especially in the case of Anaplasma and Theileria—if parasites are few it is essential that further smears be examined as the disease progresses. *Babesiella berbera* parasites may, even in fatal infections, be hard to find in blood smears, so that the discovery of these parasites in a case of suspected " tick-fever " probably indicates a clinical infection.

SUMMARY

The results of examination for tick-transmitted protozoon parasites of single blood smears from 550 clinically healthy cattle, and of repeated smears from seven healthy cattle are given.

The forms of parasites found are described, and certain abnormalities of the red blood cells noted.

The findings are discussed, and it is emphasised that when only a few parasites are found it is necessary to examine more than one smear in order to establish a diagnosis.

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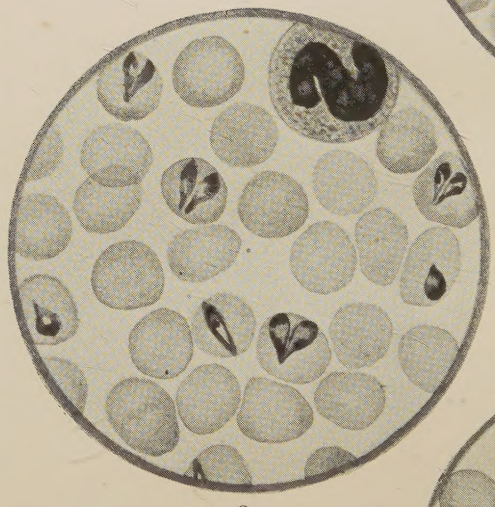

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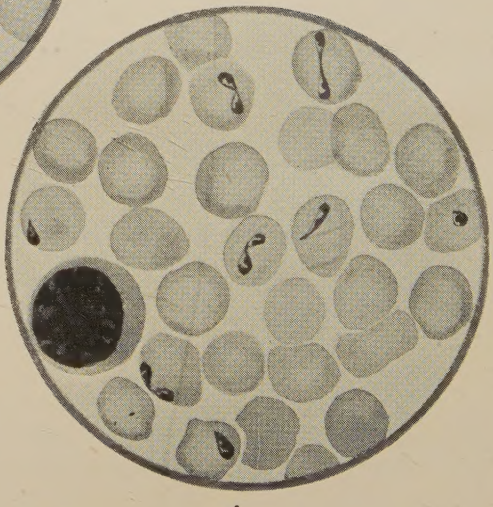

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Blood smears from cattle clinically affected with :
FIG. 1.—*Anaplasma marginale.*
FIG. 2.—*Theileria annulata.*
FIG. 3.—*Babesia bigemina.*
FIG. 4.—*Babesiella berbera.*
All × approximately 1,000

Cameron, M.B.E., manager, and Mr. M. Pfeffer, superintendent, Animal Section, of the Government Farm, and to Messrs. A. Bergthal, E. Cantor, S. Gluckstein, Y. S. Goor, A. F. Sabassi and A. Shoshan, government veterinary officers, for their willing co-operation without which this work could not have been carried out.

REFERENCES

Adler, S., and Ellenbogen, W. (1935). *Hassadeh.*, **16,** No. 1.
Sergent, E., Donatien, A., Parrot, L., Lestoquard, F., Plantureux, E., and Rougebief, H. (1924). *Ann. Inst. Pasteur*, **38,** 273.
Tchernomoretz, I. (1943). *Ann. trop. Med. Parasit.*, **37,** 77.
Wenyon, C. M. (1926). "Protozoology." Vol. II. London: Baillière, Tindall & Cox.

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STUDIES IN LOUPING-ILL

I.—CULTIVATION OF LOUPING-ILL VIRUS *IN VITRO*
II.—PATHOGENIC PROPERTIES OF CULTURE VIRUS

By

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INTRODUCTION

Studies in immunity to louping-ill were carried out under the aegis of the Animal Diseases Research Association, to determine whether tissue culture-virus could be used as an efficient substitute for sheep tissue-virus in vaccine therapy. The paper now presented as part of that investigation deals with (1) cultivation of the virus *in vitro* with the object of obtaining sufficient quantities to warrant a study of its antigenic and immunising value; and (2) observations on the pathogenic properties of culture-virus.

I.—CULTIVATION OF LOUPING-ILL VIRUS *in vitro*

By means of tissue culture technique, many viruses, including that of louping-ill, have been successfully grown *in vitro*. In the present work on the cultivation of louping-ill virus special attention has been paid to the possibility of obtaining growth in cultures of large volume and to determining, in part at least, the growth requirements of this virus. The method adopted is that described by Li and Rivers (1930) and Rivers (1931) for the cultivation of neuro-vaccine virus and dermo-virus in a medium consisting of minced viable chick embryo tissue suspended in Tyrode's solution or in a mixture of Tyrode's solution and serum, and later used by Rivers and Ward (1933) for the cultivation of louping-ill virus. No alterations in the type of medium used by Rivers and Ward were introduced save that whole chick embryos were used instead of chick embryo brain or embryos without brain, and when serum was included in the medium normal sheep serum was used instead of monkey serum.

TECHNIQUE AND MATERIALS

Tyrode's solution.—Tyrode's solution was made up in bulk as follows: NaCl, 8 gm.; KCl, 0·2 gm.; $CaCl_2$, 0·2 gm.; $MgCl_2$, 0·1 gm.; NaH_2PO_4, 0·05 gm.; $NaHCO_3$, 1·0 gm.; Glucose, 1·0 gm.; glass distilled water, 1,000 c.c.

Included in the first 12 culture passages were seven large volume cultures in all of which sheep serum was incorporated in the fluid portion of the medium in the proportion of serum 1 part, Tyrode's solution 9 parts. The mixture was sterilised by filtration using Seitz E.K. asbestos films.

Collection of serum.—Serum was obtained from sheep from non-louping-ill districts since serum from louping-ill immune animals is quite unsuitable for the present purpose. It was generally used soon after being collected but was occasionally stored at 0° to 4° C. for one to two weeks before being added to the Tyrode's fluid in the required proportion.

Tissue.—Embryonic tissue was obtained from eggs incubated nine to eleven days at 39° to 40° C. The eggs were opened under conditions designed to ensure sterility in a tissue culture box as described by Eagles and McClean (1929). Embryos were triturated to a pulp using a flat bottom specimen tube and a glass beaker; for large volume cultures mincing was accomplished with the turbo-reactor machine, the mincing mechanism of which was sterilised along with the glass jar used for collecting the embryos.

Virus.—The virus used was obtained originally from case 1930-1 (Greig, *et al.*, 1931) and had been maintained by serial passage in sheep; it had been stored *in vacuo* in the cold room in the form of dried sheep brain for periods of four or five months. Dried brain suspended in saline-broth (1/100) was centrifuged for half an hour at 3,500 r.p.m., the supernatant fluid was then filtered through kieselguhr and finally through a gradocol membrane of 0·69μ A.P.D. The virus filtrate thus obtained was used to initiate the series of cultures.

Culture flasks.—Triangular Roux culture flasks of 700 ml. capacity were used for small volume cultures, and cylindrical type flasks of 20 litre capacity (diameter 28 cm., height to shoulder 33 cm., mouth 3 cm., approximate measurements) for large volume cultures.

Setting up of cultures.—(*a*) Small volume. Cultures of 50 c.c. volume were maintained for routine virus passage. To form a first passage culture, 50 c.c. serum-Tyrode fluid, 0·5 c.c. minced chick embryo tissue and 2 c.c. virus filtrate were pipetted into each Roux flask, the cotton wool plug replaced and securely covered with parchment paper. Incubation in the flat position was carried out at 37° C. for three or four days. When the Roux flasks were laid flat the fluid formed a thin layer completely covering the lower surface. Subcultures were made by transferring 2 c.c. from one culture to 50 c.c. freshly prepared medium.

(*b*) Large volume cultures. The serum-Tyrode fluid was sterilised by filtration through two Seitz E.K. asbestos films of 14 cm. diameter into the flask in which the culture was to be made. Minced chick embryo tissue and 10 to 25 c.c. inoculum in the form of culture-virus from a Roux flask were then added by means of pipettes. The flasks were incubated on their sides or, when necessary, at a slight angle to prevent wetting of the cotton-wool plugs.

Sterility tests.—Horseflesh broth, plain agar and blood agar slopes were seeded with a few c.c. virus cultures as a test for bacterial sterility. Few of the serial passage cultures became contaminated but the proportion was greater in the case of cultures of large volume.

Determination of virus titres of cultures.—Samples for titration were withdrawn after the cultures had been shaken to detach the film of tissue cells which adheres to the glass surface. The small amount of tissue was allowed to sediment after which progressive dilutions from the supernatant fluid were made in normal saline. The presence or absence of virus in each dilution was determined by the intracerebral injection of 0·05 c.c. in mice. The injections were made immediately after the dilutions were made up to obviate the harmful or toxic effects which saline solutions are known to exert on this virus (Schwentker *et al.*, 1933).

EXPERIMENTAL RESULTS

In the course of six serial culture passages in 50 c.c. volumes of the medium, the titre of the virus, as determined by mouse intra-

TABLE I
TITRATIONS OF LOUPING-ILL VIRUS CULTURES IN MICE

Virus Passage	*Volume of Culture (Serum-Tyrode)*	*Weight Embryonic Tissue and Ratio to Fluid*	*Inoculum*	*Bacterial Sterility*	*Period of Incubation*	*Dilution of Culture*					
						0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
7th	2,000 c.c.	20 gm. 1 : 100	10 c.c. 6th passage virus	Negative	Pre-incubation	8, 8	9, 9	S, S	S, S	S, S	S, S
					1 day	8, 10	8, 10	S, S	S, D	S, S	S, S
					4 days	6, 6	6, 6, 6	6, 6, 7	7, 8, 8	8, 9, S	S, S, S
					6 days	5, 6, 6	6, 6, 7	7, D, D	10, 11, 11	11, 11, 12	S, S, S
					8 days	5, 6, 6	6, 6, 6	6, 7, D	8, 9, 9	8, S, S	S, S, S
8th	7,000 c.c.	34 gm. 1 : 206	10 c.c. 7th passage virus	Negative	5 days	6, 6, 6	6, 6, 7	7, 9, 9	9, S, S	S, S, S	S, S, S
9th	10,000 c.c.	52 gm. 1 : 200	10 c.c. 8th passage virus	Negative	4 days	5, 5, 6	6, 6, 7	D, 7, 8	8, 9, 9	10, 10, S	S, S, D
10th	9,000 c.c.	60 gm. 1 : 150	10 c.c. 9th passage virus	Negative	19 hours	9, 9, S	12, S, S	S, S, S	S, S, S	—	—
					43 hours	6, 6, 6	5, 6, 7	8, 8, 9	S, S, S	S, S, S	S, S, S
					4 days	6, 6, 6	7, 7, 8	6, 7, 8	9, 9, S	S, S, S	S, S, S
10th	7,000 c.c.	50 gm. 1 : 140	10 c.c. 9th passage virus	Negative	42 hours	6, 6, 6	7, 7, D	7, 7, 8	9, 10, 10	S, S, S	S, S, S
					4 days	5, 6, 6	6, 6, 7	7, 7, 7	9, 9, 11	S, S, S	S, S, S
11th	12,000 c.c.	143 gm. 1 : 84	10 c.c. 10th passage virus	Negative	38 hours	6, 7	7, 7, 7	6, 7, 7	D, S, S	D, S, S	S, S, S
					3½ days	5, 5	6, 7, 7	7, 7, 7,	7, 8, 9	13, 15, S	S, S, S
12th	14,000 c.c.	130 gm. 1 : 108	25 c.c. 11th passage virus	Negative	3½ days	5, 5, 6	7, 7, D	7, 7, 7, 7, 7	8, 8, 8, 8, 9, 11	9, 10, 10, 11, 12, S	S, S, S, S

Figure = destruction of the mouse when showing typical symptoms of louping-ill *x* days after inoculation.
D = death from undetermined cause sometimes occurring a few days after inoculation and sometimes within the period in which louping-ill could have been expected to develop.
S = survived for a period of 15 days.

cerebral test, was positive at a 10^{-3} or a 10^{-4} or, on occasion, a 10^{-5} dilution of culture. Mice which became affected usually developed typical paralyses in five to eight days (Alston and Gibson, 1931, and Greig, *et al.*, 1931) but occasionally characteristic symptoms of louping-ill were not manifest until 9 to 14 days after inoculation. As a rule this delay occurred only when the inoculum had consisted of the higher dilutions of culture (10^{-4} or 10^{-5}). The syndrome did not differ from that observed in mice which had received sheep brain-virus and death usually took place in 24 to 48 hours following the appearance of the first symptoms.

Apart from volume and the consequent variation in depth of fluid covering the tissue cells, the conditions under which these cultures were incubated were the same as those for 50 c.c. volume cultures in that identical serum-Tyrode solution was used and the ratio of tissue to fluid employed was reasonably proportionate. Seven cultures varying from 2 to 14 litres in volume and representing 7th to 12th culture passage-virus, were examined. Details and infective titres are given in Table I.

In the seventh passage culture (Table I) no demonstrable increase in the virus content was detected after an incubation period of 24 hours ; during the following three days a marked rise took place, and apparently reached a maximum, since no further increase occurred up to the eighth day, when incubation was discontinued. This increase during the second to fourth day was further demonstrated in one of the tenth and in the eleventh passage cultures, where higher titres were obtained on the fourth than on the second day. Of the remaining four cultures, three were tested once only —at the end of a three- to five-day period of incubation ; in two the high end point of infectivity suggests that a similar rise must have occurred, whilst the end point titre of 10^{-3} in the remaining two does not necessarily indicate absence of increase, in view of the demonstrated increase in tenth culture passage which had a similar end point.

OBSERVATIONS ON THE REQUIREMENTS OF LOUPING-ILL VIRUS FOR GROWTH *in vitro*

It is generally accepted that *in vitro* cultivation of a virus is possible only in the presence of tissue cells which survive over a suitable period of time ; indeed, those with experience of this type of work regard with favour cultures which do not only permit survival of cells but show definite evidence of cellular activity or metabolism. The observations on growth requirements of louping-ill virus recorded here have been limited to the *p*H reaction of the medium and minimum quantity of chick embryo tissue which would yield maximum titres of virus, although it is realised that these aspects border only on the fringe of the determination of possible optimum requirements.

pH Reaction of the Medium

There is a fairly wide range (approximately *p*H 5 to *p*H 9) of acidity or alkalinity in which tissues will remain active (Lewis and Felton, 1922, cited by Willmer, 1935). A knowledge of the growth requirements *in vitro* was essential since the full range might not be suitable for multiplication. Early observations showed that the reaction of Tyrode's solution, when made up according to the formula given on page 250, was *p*H 8·2 to *p*H 8·4. Changes, however, took place following the addition of sheep serum, of embryonic tissue cells, and during the period of incubation when active cellular metabolism was in progress. As an example of the sequence of changes the electrometric readings obtained from the 14,000 c.c. culture (Table I) may be cited : Tyrode's solution, *p*H 8·36 ; after addition of sheep serum, 8·14 ; after addition of tissue cells, 7·98 ; after 1 day 14 hours' incubation, 7·62, and after 3 days 14 hours' incubation, 7·63.

Additional observations were made with cultures of 50 c.c. volume using the same medium with the exception that serum was omitted since it had already been determined that equally good virus titres could be obtained when the fluid portion consisted of Tyrode's solution alone. Samples of Tyrode's solution were adjusted colorimetrically to reactions of *p*H 5, 6, 7, 7·2, 7·4, 7·6, 7·8, 8, 8·2, 8·4 and 9 by the addition of the required quantity of a 5 per cent. solution of phosphoric acid (H_3PO_4) or N/1 NaOH. In view of the difficulty of obtaining Tyrode's fluid which would maintain for long the more acid reactions all adjustments, filtrations and setting up of cultures were made on the same day. Virus titres were determined by mouse test after the cultures had been incubated for approximately four days at 37° C. The dilutions were made in Tyrode's solution (*p*H 8·2) instead of in saline.

TABLE II

EFFECT OF VARYING THE INITIAL *p*H REACTION OF CULTURES. VIRUS TITRATION IN MICE

Initial pH *Reaction of Culture*	*Dilution of Culture*					
	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
9	—	6, 6, 6	6, 7, 7	7, 8, 8	8, 8, S	S, S, S
8·4	—	—	6, 6, 7	7, 7, 8	7, 8, 9	11, 13, S
8·2	—	—	7, 7, 8	7, 8, 8	8, 9, 10	9, 10, 11
8	—	—	7, 7, 7	7, 7, 7	8, 8, 9	9, 10, S
7·8	—	—	6, 6, 7	6, 8, 10	9, 9, 11	9, D, S
7·6	—	—	5, 6, 7	D, 7, 8	9, 9, 9	10, D, S
7·4	—	—	D, 6, 6	7, 7, 7	D, 10, 10	10, S, S
7·2	—	—	6, 6, 8	6, 6, 9	D, D, S	9, D, S
7	—	—	7, 7, 8	8, 8, 8	D, 8, 10	—
6	S, S, S	S, S, S	S, S, S	S, S, S	D, S, S	—
5	S, S, S	S, S, S	D, S, S	S, S, S	S, S, S	—

Abbreviations as in Table I.

In two experiments the titration results indicated that while initial reactions of Tyrode's fluid of *p*H 7 to *p*H 9 were suitable for the growth of louping-ill virus, reactions of *p*H 5 and *p*H 6 were decidedly harmful since virus not only did not multiply but failed to survive. In a third experiment the same result was obtained with the difference that the culture with an initial reaction of *p*H 6 contained virus in a dilution of 10^{-3}. Considerable change in the reaction had, however, taken place, for when tested at the termination of the period of incubation a reading of *p*H 6·8 was obtained. Apparently virus had survived an initial exposure to the H ion concentration of *p*H 6 and following improvement in the environmental conditions multiplication had taken place.

Ratio of Tissue to Fluid

For these experiments 1 c.c. of chick tissue was taken to be the equivalent of 1 gm. and appropriate amounts were added to culture flasks each containing 50 c.c. Tyrode's solution (*p*H 8·2) to form a scale of ratios of 1 : 800, 1 : 400, 1 : 200, 1 : 133, 1 : 100, 1 : 66, 1 : 50, 1 : 33, 1 : 25. Virus was added and titration of the contents of a few flasks before incubation was commenced formed a base line from which it was later judged whether virus had increased during the period of incubation.

Virus in titre which apparently represents the maximum obtainable in this medium was present when the ratio of chick tissue to Tyrode's fluid lay between 1 : 100 and 1 : 33. Cultures which contained 4 per cent. concentrations of tissue did not at any time yield titres of virus equal to those found in cultures whose tissue to fluid ratio ranged between 1 : 100 and 1 : 33. Cultures with minimum concentrations of tissue (1 : 800 and 1 : 400) while able to support survival of virus showed no definite evidence of multiplication which if growth had taken place could only have done so to a very slight degree.

Comparative Sensitivity of Tissue Culture Medium and Mice to Louping-ill Virus

In the routine serial passaging of louping-ill virus in cultures of 50 c.c. volume the titre of virus, resulting from the quantity of virus inoculum customarily used, was very frequently positive by mouse test at dilutions of 10^{-2} before and 10^{-4} or 10^{-5} after incubation respectively. From a practical point of view and more especially with regard to cultures in volumes of several litres it was important to determine whether smaller quantities of virus as inoculum would result in the development of satisfactory virus titres and whether there were any reasons for suspecting the sensitivity of a medium of this type to " infection " with louping-ill virus. Three separate samples were titrated consisting of a gradocol membrane filtrate (1) prepared from a broth-saline suspension of sheep brain-virus, the

TABLE III

EFFECT OF VARYING THE RATIO OF TISSUE TO FLUID IN CULTURES. VIRUS TITRATIONS IN MICE

Ratio of Tissue to Fluid in Culture	*Dilution of Culture*						
	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1 : 25	—	—	7, 7, 8, 8	7, 7, 8, 9	8, 9, 10, 11	D, S, S, S	S, S, S, S
1 : 33	—	—	7, 7, 7, 8	7, 8, 8, 9	8, 8, 9, 12	10, 14, S, S	S, S, S
1 : 50	—	—	—	7, 8, 8, 8	8, 8, 9, 9	10, 13, S, S	D, S, S, S
1 : 66	—	—	—	7, 7, 8, 9	7, 8, 8, 9	12, 15, S, S	11, S, S, S
1 : 100	—	—	—	8, 8, 8, 9	D, 8, 10, S	13, S, S, S	S, S, S, S
1 : 133	—	—	—	8, 9, 10, 12	D, S, S, S	S, S, S, S	S, S, S, S
1 : 200	—	—	D, 7, 8, 9	10, 10, S, S	10, S, S, S	S, S, S, S	S, S, S
1 : 400	7, 8, 8	7, 8, 9, 9	8, 12, S, S	S, S, S, S	S, S, S, S	—	—
1 : 800	7, 8, 8, 8	8, 8, 8, S	13, S, S, S	S, S, S, S	—	—	—
Average pre-incubation titre	6, 7, 7, 8, 8	7, 8, 8, 9, 9	9, 9, 11, S, S	S, S, S, S, S	—	—	—

Abreviations as in Table I.

TABLE IV
COMPARATIVE TITRATION OF LOUPING-ILL VIRUS IN MICE AND IN CULTURE.
(SUBINOCULATION OF MICE WITH UNDILUTED CULTURE AS A TEST FOR PRESENCE OR ABSENCE OF VIRUS)

Virus Sample	*Test Organism*	*Dilution of Virus used in Inoculum*								
		0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
(1)	Mouse	9, 10, 10 11, S, S	S, S, S, S, S, S	D, S, S, S, S, S	S, S, S, S, S, S	D, S, S, S, S, S	S, S, S, S, S, S	—	—	—
	Culture	5, 5, 6, 6, 6	5, 6, 6, 6, 6	4, 5, 5, 6, 6	5, 6, 6, 6, 6	S, S, S, S, S	D, S, S, S, S	—	—	—
(2)	Mouse	—	—	—	10, 13, S, S	D, S, S, S	S, S, S, S	D, S, S, S	S, S, S	—
	Culture	—	—	—	5, 6, 6, 7	6, 6, 6, 6	S, S, S, S	S, S, S, S	S, S, S, S	—
(3)	Mouse	—	—	6, 7	7, 7, 7	7, 7, 7, 7, 8	7, 8, 9, S, S, S	9, S, S, S, S, S	S, S, S, S, S, S	S, S, S, S, S, S
	Culture	—	—	—	4, 5, 5, 5	5, 5, 5, 5	5, 5, 5, 5	4, 5, 5, 5	S, S, S, S	S, S, S, S

Abbreviations as in Table I.

filtrate having been stored in the cold room for 18 hours pending the result of a sterility test before titration was carried out, and two culture virus samples (2 and 3).

In each instance progressive virus dilutions were made with the usual precautions in normal sheep serum or, in the case of one virus sample (2) in Tyrode's solution. A culture in freshly prepared medium of chick tissue in Tyrode's solution was then made from each dilution using 2 c.c. as inoculum. Immediately afterwards mice were inoculated intracerebrally with the same dilutions. After incubation periods of approximately four days each culture was tested for presence or absence of virus. The results which are shown in Table IV appear to indicate that tissue culture-medium is to be preferred as a means of detecting the presence of minimum quantities of louping-ill virus in a given suspension, though it was not very much superior in the case of sample (3). This observation might indicate some degree of insensitivity of the mouse to infection with louping-ill virus, but it should be remembered that 2 c.c. virus dilution was used as inoculum for cultures as against 0·05 c.c. as inoculum for mice. If the possibility of detecting virus in a particular sample were directly proportional to the quantity used as inoculum then in these tests the chance in favour of a culture as compared with a single mouse would be as 40 : 1. If the view of possible deterioration in the pathogenic properties for the living animal, perhaps as a result of the effects of filtration or of the period of storage and exposure to saline-broth sample (1), is not accepted, then it is obvious that 25 times more virus was required to infect a mouse than to " infect " chick tissue culture-medium. With the two culture-virus samples which were not subjected to the same possibly harmful influences, the culture media were apparently only 0·25 times more sensitive to inoculation than were mice.

It was shown in the examination of virus sample (2) and in lesser degree in virus sample (3) that minimum quantities of virus in a suspension can be more readily demonstrated by primary inoculation of a culture followed by subinoculation of mice than by the direct test in the mouse. In sample (2) the end point of infectivity to mice was reached at a 10^{-3} dilution, since two out of four mice developed typical louping-ill whereas no symptoms developed in those receiving a 10^{-4} dilution. Nevertheless, a 10^{-4} dilution did contain virus, since its presence was readily demonstrated by subinoculation of mice with a tissue culture which had received 2 c.c. of this dilution as inoculum. No difference in the end point to infectivity for mouse and culture was observed in the case of virus sample (3), but it should be noted that three only out of six and one only out of six mice which received 10^{-5} and 10^{-6} dilutions respectively developed louping-ill, whereas all the mice inoculated from the culture of these dilutions died. Further, judging by the incubation periods of four to five days necessary for the development of louping-ill in these mice, it is considered that virus was present in considerable titre in

both cultures. This conclusion has been borne out by repeated testing of cultures. It has usually been observed that if mice injected intracerebrally with undiluted culture develop typical symptoms of louping-ill following a short incubation period of four or five days, then virus in considerable titre is present in the culture.

Viability of Louping-ill Culture-Virus

As already recorded (page 254), while alteration of the *p*H index is to be expected during the preparation and period of incubation of cultures of chick embryo tissue in Tyrode's solution, the reaction at the end of the period has very frequently been found in the region of *p*H 7·6 to *p*H 7·8. Moreover, during long periods of approximately 100 days of storage at 0° to 4° C., the reactions of cultures have remained within the same limits of *p*H 7·6 to *p*H 7·8.

By the method of subculture at ten-day intervals, virus in a 15th passage culture was found by mouse test to remain active for 60 days but not for 70 days. In a second experiment a number of cultures selected for subcultivation were found to be active after storage for the following periods: 82 days (13th), 99 days (40th), 82 days (42nd). One culture (tenth passage) was inactive after storage for 105 days.

The number of passages in culture, therefore, did not appear to influence the viability of louping-ill virus during storage at 0° to 4° C. In the first experiment active virus was present after 60 days but not after 70 days. In the second where active virus was present for 99 days but not after 105 days, the parent cultures had not been removed from the cold room to the changed conditions of the laboratory during the period of storage. This in some measure may account for the shorter survival period observed in the first experiment when the parent cultures were removed from the cold room on several occasions for periods of five to ten minutes.

DISCUSSION

If the flasks containing the culture are stood upright the vertical surface supports only minimum quantities of the very minute particles of tissue; after incubation these are found adhering to the glass as small foci of active cells scattered irregularly and sparsely. To obtain the greatest available area of glass surface as support for the active tissue cells, the 20-litre flasks used for cultures of 2,000 c.c. to 14,000 c.c. volume were incubated on their sides or, when the volume was in excess of 8,000 c.c., at an angle to prevent wetting of the cotton-wool plugs. In the upright position the area of the base of the flask was approximately 615 sq. cm. ($3{\cdot}14 \times 14^2$), whereas when on its side the available supporting surface was approximately 1,452 sq. cm. $\left(\frac{3{\cdot}14d \times 33}{2}\right)$ less a small but undeterminable amount for the vertical and almost vertical part of the curving sides.

The difference in the available surface was therefore considerable. When at a slight angle, the base, now removed from the vertical, was available in proportion to the area which became covered with fluid. With a 10,000 c.c. culture, nearly all of the base area (615 sq. cm.) and of the sides (1,452 sq. cm.) which supported the fluid was available to the tissue cells. Although the method resulted in varying depths of fluid covering the cells in each culture and also in the separate cultures depending on their volume, cellular activity did not appear to be more pronounced in the shallower parts, *i.e.*, to be dependent on nearness of the cells to atmospheric oxygen, for a film or syncytium of tissue cells adhering to the supporting glass formed irrespective of the depth of fluid above them. Excepting in the culture of 2,000 c.c. volume the tissue cells were approximately 12 cm. from atmospheric air in the deepest part, and when incubation was carried out at an angle this distance was frequently greater. Judging by the virus titres obtained, the environmental conditions suitable for virus propagation were as satisfactory in the large volume cultures as in those of 50 c.c. volume in Roux flasks where the fluid layer covering the tissue cells was estimated to be 4·5 mm. deep. Tissue cells from ten-day-old chick embryos have mainly been employed in this work; the possibility of a different result with older cells has not been determined but with this reservation it is interesting to note the discrepancy of the above result with that of Maitland, Laing and Lyth (1932), who, in their work on cultivation of vaccinia virus *in vitro* in a medium of minced adult rabbit kidney and Tyrode's fluid found that ready access of oxygen to the tissue in the medium was essential for growth of the virus and that when the tissue was covered by a layer of liquid 15 mm. deep survival of cells was insufficiently long to allow of virus multiplication taking place.

With louping-ill virus the expectation of obtaining good virus titres appears dependent, in part at least, on the ratio of chick tissue to Tyrode's solution employed and not on volume of the culture. When cultivated in 50 c.c. volumes the evidence obtained indicated that in all probability a tissue to fluid ratio of 1 : 66 (1·5 per cent. tissue) represented the minimum quantity of tissue which would yield maximum titres of virus. Three of the four large volume cultures which were positive at a 10^{-4} dilution contained tissue concentrations nearest to this ratio (1 : 100, 1 : 84 and 1 : 108, Table I).

The changes occurring in the reaction in the different stages of preparation and incubation of cultures, although inevitable, are apparently not unsatisfactory for the growth requirements of louping-ill virus. It appeared that the alkaline side of neutrality was the most favourable since virus propagated apparently to maximum titre in cultures with initial reactions of *p*H 7 to *p*H 9. In contrast, louping-ill virus did not survive, except in one experiment already commented on, in cultures with an initial reaction of *p*H 6·0, although this range still permitted tissue metabolism.

Chick embryo tissue and Tyrode's fluid, however, has never failed to detect particularly small quantities of virus in a dilution which has been infective for only a proportion of mice inoculated directly. In some instances primary inoculation of the tissue-fluid medium, followed, after suitable incubation, by subinoculation of mice, detected virus in suspensions which were negative on direct test in mice. The sensitive nature of the medium may resemble that of the chorio-allantoic membrane of the developing egg, found by Burnet (1936) to be significantly more sensitive to infection by louping-ill virus than the mouse brain ; on the other hand, it may be a simple physical expression of the increased chances, at very high dilutions, of including a single infective unit in the larger random sample represented by the culture inoculum in comparison with the mouse inoculum.

SUMMARY

This work has confirmed the observations of Rivers and Ward that a medium of chick embryo tissue in Tyrode's solution to which 10 per cent. serum had been added is suitable for *in vitro* cultivation of louping-ill virus. Serum may be omitted from the medium without appreciably affecting the activity of the tissue cells or the propagation of virus.

The same medium in 2,000 c.c. to 14,000 c.c. quantities is equally suitable and massive amounts of virus can be obtained by this means. Within the limits tested the volume of a culture or the distance of the contained tissue from free oxygen does not appreciably interfere with the ability of ten-day-old chick embryo cells to support the multiplication of louping-ill virus *in vitro*.

The titre of virus which may be expected to develop depends on the ratio of tissue to fluid employed, the optimum ratio falling between 1 : 100 and 1 : 33.

Conditions favourable for the cultivation of louping-ill virus are found in a range of *p*H 7 to *p*H 9. Acid media with reactions in the neighbourhood of *p*H 6 are decidedly harmful.

The medium is sensitive and may be used to confirm the presence of particularly small quantities of virus.

Louping-ill virus in culture survives in the cold room at 0° to 4° C. for periods of up to 99 days.

II.—OBSERVATIONS ON THE PATHOGENIC PROPERTIES OF CULTURE-VIRUS

Although culture passage-virus had shown no evidence of instability in so far as its neurotropic characteristics for mice were concerned, it was necessary to determine what changes, if any, had occurred with respect to the viscerotropic properties for sheep possessed by non-passage virus.

Material from the 20th, 40th, 60th and 170th passage cultures, this last representing serial passage over a period of six years two

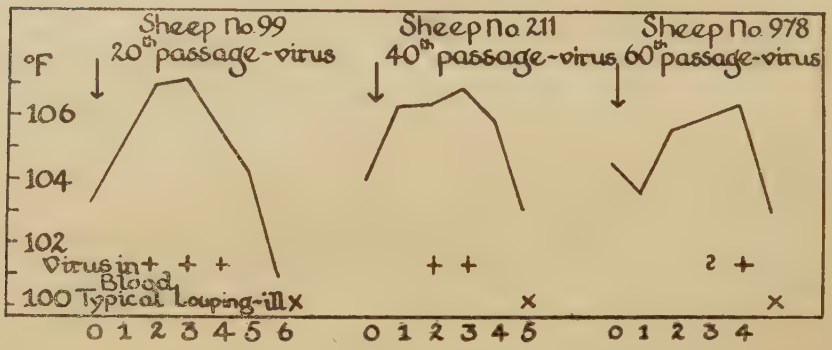
months, was injected into sheep by either the intracerebral or the subcutaneous route.

Intracerebral Injection

Sheep Nos. 99, 211, 978 and 351, which received 1·0 c.c. whole culture-virus of 20th, 40th, 60th or 170th passage respectively, developed louping-ill. In each animal the syndrome exhibited was clinically indistinguishable from that resulting from a corresponding injection with brain-virus. The temperature rose appreciably 24 to 48 hours after virus was injected (*vide* Chart 1), continued to rise

Chart 1

Temperature Curves of Sheep after Intracerebral Inoculation with Louping-ill Culture Passage-virus



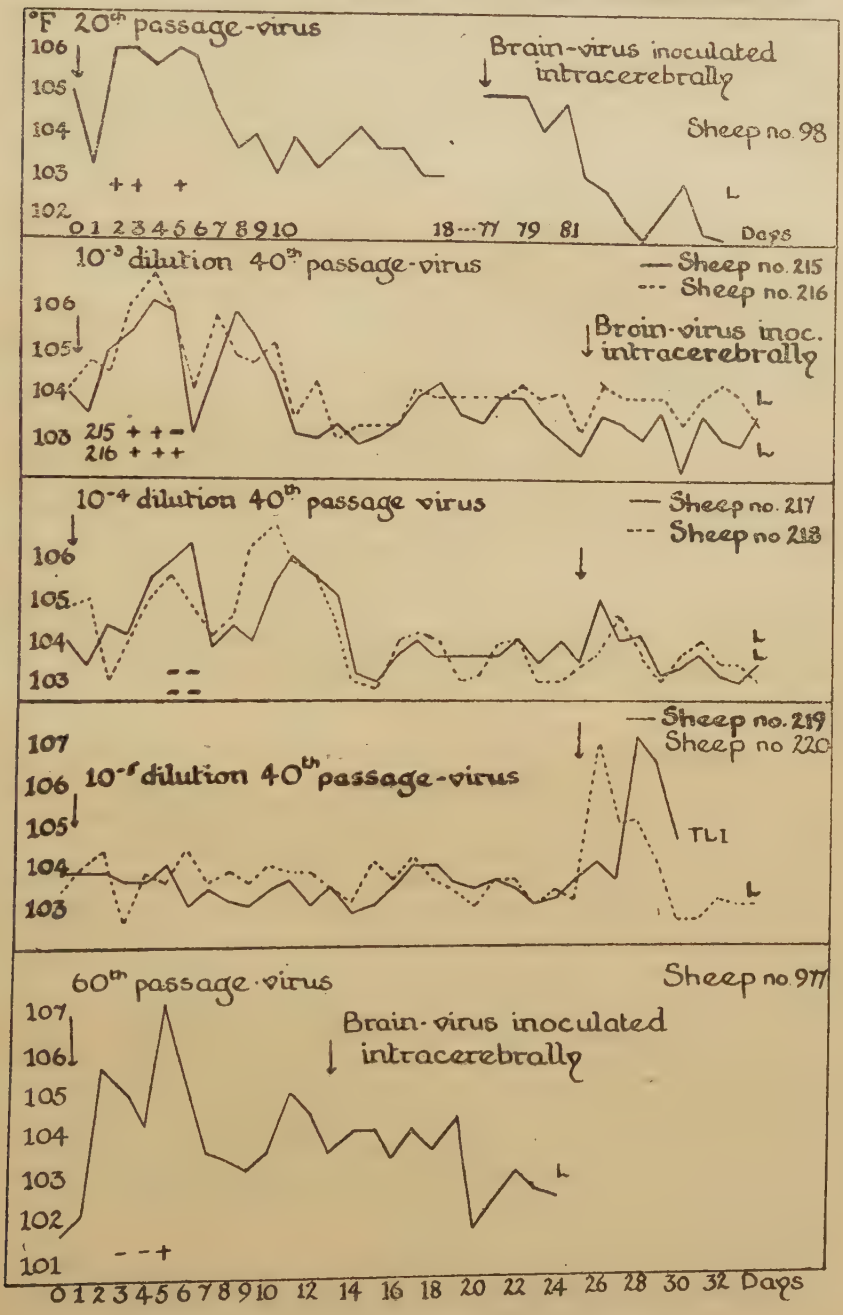
for a further two or three days to 106° F. or over, and thereafter fell when the first symptoms of inco-ordination in gait were observed. As with brain-virus the syndrome progressed rapidly and by the fifth day all four animals suffered from severe ataxia and were unable to stand. The animals were destroyed six, five, five and six days respectively after inoculation, soon after the development of coma, which ensued in each case.

Subcutaneous Injection in Sheep

The pathogenic property of virus of the four passages under examination was apparently unimpaired. A culture from the 40th passage which infected mice on intracerebral injection at a dilution of 10^{-4} was infective on subcutaneous injection in sheep at the same dilution. In ten of the sheep (Nos. 98, 212, 213, 214, 215, 216, 217, 218, 977, and 354) a period of fever commenced usually on the second day following inoculation ; with the exception of sheep No. 213, which exhibited a high temperature on one day only, the febrile period in all others persisted for several days and frequently was diphasic in nature (Chart 2). No symptom other than those which could be associated with fever was observed in any of the test animals.

CHART 2

TEMPERATURE CURVES OF SHEEP AFTER SUBCUTANEOUS INOCULATION WITH LOUPING-ILL CULTURE PASSAGE-VIRUS AND SUBSEQUENT TEST FOR DEVELOPED IMMUNITY



Abbreviations : + = virus present in the blood-stream.
— = virus not detected.
L = lived.
TLI = typical louping-ill.

Blood samples obtained from the ten animals which exhibited thermal response and from the four which received passage-virus by the intracerebral route, were tested for presence of virus by intracerebral injection of 0·05 c.c. doses into mice. Frequently, examination of two or three samples of blood drawn on consecutive days during the febrile period of each animal was made. Virus was present on one or more days in the blood-stream of eight of the ten reactors inoculated subcutaneously ; there was, however, some doubt as to its ever having appeared in the blood of the remaining two. In all four animals injected intracerebrally virus appeared in the blood-stream and at *post-mortem* examination the spleen tissue contained virus in a titre comparable to that found in the spleens of sheep receiving brain-virus by the intracerebral route.

None of the samples of blood was examined in a dilution greater than 10^{-1}, but it is possible that in those cases in which all the mice developed louping-ill after incubation periods of six to eight days, virus could still have been demonstrable in higher dilutions. Its presence in a 10^{-1} dilution indicates 200 mouse infective doses of virus per c.c. of circulating blood and in total the amount of virus in certain of the sheep must have been considerably in excess of the amount of culture-virus which was injected. Sheep 215 and 216 received 5 c.c. of a 10^{-3} dilution of the culture which was found infective for mice in doses of 0·05 c.c. at a 10^{-4} dilution but not at a 10^{-5} dilution ; they received, therefore, 1,000 mouse infective doses of virus, a quantity which must certainly have been less than the estimated amount in the blood during the phase of active infection.

DEVELOPMENT OF IMMUNITY FOLLOWING INFECTION WITH PASSAGE-VIRUS

The various changes, including the development of immunity which follows the subcutaneous injection of brain-virus into sheep, were described by Greig, *et al.* (1931). Sheep which have survived the infection with or without symptoms of brain or spinal cord dysfunction resist subsequent introduction of virus into the central nervous ssytem in doses lethal to non-immune sheep, *i.e.*, they develop no manifest symptoms. The immunity resulting from the subcutaneous injection of culture passage-virus was similarly determined using brain-virus in an amount approximating to 20,000 mouse infecting doses for the intracerebral test injections. The intervals between the experimental infection and the subsequent immunity-test dose varied, in the order of the cultures used, these being 77, 25, 13 and 18 days. Temperature records of the animals were obtained (Chart 2). A summary of the experiments is set out in Table V.

Taken in conjunction with the result of the previous experiment —the development of a diphasic febrile phase with the appearance of virus in the blood-stream—it seems certain that passage serially

Table V
Pathogenicity of Culture-Virus for Sheep

Sheep No.	Virus Passage	Dose and Method of Inoculation	Result		Interval between Inoculations (in Days)	Test for Immunity to Non-passage (Brain) Virus. Inoculations made Intracerebrally		
			Thermal Response	Virus in Blood		Dose	Result: Thermal Response	Result: Clinical Symptoms
98	20th	5 c.c. Sc.	+	+	77	20,000 M.I.D.	—	None
99		1 c.c. Ic.	+ T.L.I.	+				
211		1 c.c. Ic.	+ T.L.I.	+				
212		5 c.c. 10^{-1} Sc.	+	+	25	20,000 M.I.D. (approx.)	—	None
213		5 c.c. 10^{-2} Sc.	+	+	25	"	—	"
214		5 c.c. 10^{-2} Sc.	+	+	25	"	—	"
215		5 c.c. 10^{-3} Sc.	+	+	25	"	—	"
216	40th	5 c.c. 10^{-3} Sc.	+	+	25	"	—	"
217		5 c.c. 10^{-4} Sc.	+	?	25	"	—	"
218		5 c.c. 10^{-4} Sc.	+	?	25	"	—	"
219		5 c.c. 10^{-5} Sc.	—	Not tested	25	"	+	T.L.I.
220		5 c.c. 10^{-5} Sc.	—	"	25	"	+	Survived
221		5 c.c. 10^{-6} Sc.	—	"				
222		5 c.c. 10^{-6} Sc.	—	"				
977	60th	5 c.c. Sc.	+	+	13	20,000 M.I.D. (approx.)	—	None
978		1 c.c. Ic.	+ T.L.I.	+				
351	170th	1 c.c. Ic.	+ T.L.I.	+				
354		5 c.c. Sc.	+	+	18	20,000 M.I.D.	—	None

Abbreviations: Sc. = subcutaneous. Ic. = intracerebral. M.I.D. = Mouse infecting doses. T.L.I. = typical louping-ill.

170 times in culture fails to modify the pathogenic character or the antigenic structure of louping-ill virus. All those animals which exhibited thermal reactions subsequent to subcutaneous injection of culture passage-virus survived intracerebral injection of brain-virus. Of the two sheep which had received 5 c.c. of a 10^{-5} dilution of the 40th passage culture and showed no subsequent thermal reaction, one (No. 219) developed typical louping-ill five days after the intracerebral injection, while the other (No. 220) unexpectedly showed only a temperature rise and survived the secondary injection.

HISTOLOGY

The histological changes found in the central nervous tissues of sheep (Nos. 99, 211, 978 and 351) succumbing to intracerebral injection of passage-virus varied little from case to case and, in general, resembled those produced by louping-ill virus on the nerve tissues of this species. In comparison with the lesions of experimental louping-ill as described by Brownlee and Wilson (1932) the meningeal, vascular and interstitial changes were less prominent while, in further contrast, the damage to the nerve cells was more severe and widely distributed in each of the four sheep. Thus, perivascular infiltrations and focal accumulations of microglial cells were not readily recognised in any part of the central nervous system. The large nerve cells, particularly those in the medulla and in the grey matter of the spinal cord suffered acute necrosis, and reactive change, such as neuronophagia, was absent or developed only in very mild form. In the main the nature of the lesions resulting from brain-virus and from passage-virus were similar and the distinctions here referred to are regarded only as variations in the degree of the pathological changes in the special cells and structures involved.

SUMMARY

Louping-ill virus passaged serially 170 times in medium consisting of chick embryo tissue plus Tyrode's fluid, retains its viscerotropic characteristics for sheep and its neurotropic characteristics for both sheep and mice.

Infection in sheep resulting from subcutaneous injection of culture-virus stimulates the development of an immunity similar to that existing in survivors of infection with brain-virus.

Following intracerebral injection of culture-virus into sheep, the most striking pathological changes occurred in the nerve cells indicating a direct attack by virus on these elements.

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REFERENCES

Alston, J. M., and Gibson, H. J. (1931). *Brit. J. exp. Path.*, **12,** 82.
Brownlee, A., and Wilson, D. R. (1932). *J. comp. Path.*, **45,** 67.
Burnet, F. M. (1936). *Brit. J. exp. Path.*, **17,** 294.
Eagles, G. H., and McClean, D. (1929). *Ibid.*, **10,** 35.
Greig, J. R., Brownlee, A., Wilson, D. R., and Gordon, W. S. (1931). *Vet. Rec.*, **11** (New Ser.), 825.
Li, C. P., and Rivers, T. M. (1930). *J. exp. Med.*, **52,** 465.
Maitland, H. B., Laing, A. W., and Lyth, R. (1932). *Brit. J. exp. Path.*, **13,** 90.
Rivers, T. M. (1931). *J. exp. Med.*, **54,** 453.
Rivers, T. M., and Ward, S. M. (1933). *Proc. Soc. exp. Biol. & Med.*, **30,** 1300.
Schwentker, F. F., Rivers, T. M., and Finkelstein, M. H. (1933). *J. exp.* Med., **57,** 995.
Willmer, E. N. (1935). *Tissue Culture. Methuen's Monographs on Biological Subjects.*

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THE SEROLOGICAL DIAGNOSIS OF JOHNE'S DISEASE OF SHEEP

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INTRODUCTION

In 1916 M'Fadyean *et al.* reported the preparation of a new diagnostic product from cultures of *M. johnei* from cattle. This product, to which they gave the name johnin, has been used for diagnostic work with cattle especially since Dunkin's (1928) announcement that infected animals reacted specifically to this substance after intradermal injection. Glover (1941) has recently reported upon the concentration and purification of the active principle using the method employed by Seibert for the purification of tuberculin. It seems that this " PPD " johnin has not yet been extensively used for field work, but that the results with it so far have been promising.

Animals affected with Johne's disease are not as highly allergic towards the products of *M. johnei* as are tuberculous animals towards tuberculin, as judged by the results of intradermal tests. Much larger doses of johnin are needed to elicit a reaction than are stipulated for tuberculin, and even then a large proportion of undoubted cases of Johne's disease do not react. False negative reactions can be correlated in number with the initial and terminal stages of infection. Thus recently infected animals may fail to react, as the allergic state seems to develop slowly, and advanced cases are especially frequently found to be anergic to johnin. False positive reactions are also not uncommon. In spite of these shortcomings the intradermal test with johnin is of great importance.

The Complement Fixation Test (CFT) with sera from infected animals has been tried for the diagnosis of Johne's disease, especially by Hagan and his co-workers (Hagan and Zeissig, 1933, a, b), who used lipoid antigens prepared from cultures of *M. johnei*. Sera from infected cattle generally fixed complement in the presence of such antigens, but the reaction was reported to be insufficiently specific, in that sera from normal animals also often reacted positively. The reaction therefore has not found practical application, although Hagan believes it might be of importance in some circumstances.

We have worked in this laboratory on a complement-fixing antigen prepared from intestinal mucosa of sheep affected with Johne's disease. This antigen, which seems to be a protein, fixes complement in the presence of sera obtained from sheep infected with Johne's disease, and only rarely with sera from normal sheep and then only in low titre.

The present paper describes the results obtained in a comparative study of this new complement fixation test and the intradermal reaction with PPD. This study was carried out on a small but heavily infected flock of sheep.

METHODS

Complement Fixation Test

The technique of the complement fixation test has already been described in detail (Sigurdsson, 1945). The method of preparing the antigen used in the work here reported and the technique of the test were as follows.

The intestine having been opened, the infected mucosa was lightly washed with running water, scraped off with a knife and frozen. The frozen intestinal mucosa was minced and spread on large petri dishes which were then put in a vacuum oven heated with warm water to a temperature of 40 to 50° C. and subjected to a negative pressure of 10 to 40 mm. Hg. Several (five or more) dried intestinal mucosae containing large numbers of bacilli were ground very thoroughly in a ball mill. The light powder keeps well at room temperature.

The antigen was prepared by suspending 1 g. of powder in 60 ml. of normal saline solution. The suspension was adjusted to a *p*H of about 7·0 by adding 1N NaOH. It was kept in the cold for about 20 hours, centrifuged and the supernatant was removed.

The antigen (supernatant) should be titrated before use. Dilutions in buffered saline* were tested with sera of known strength, two to four positive sera of different strengths and two known negatives being used for each dilution of antigen.

The highest dilution of antigen that gave approximately the optimal reaction was determined, and the dilution next below was used in the main test. Sera from infected sheep were kept frozen until required for use.

It was found that, after inactivation at 50 to 60° C. for 30 minutes, sheep sera were so anticomplementary as to make them unsuitable for use. Quantitative determination of the effect of different temperatures of inactivation showed that serum diluted 1 to 4, which is the lowest dilution used in the test, showed an anticomplementary effect approximately as follows: After 30 minutes at 50° C., 4 MHDs of complement were destroyed or inhibited by serum, at 55° C., 3 MHDs, at 60° C., 1 MHD and at 65° C. no anticomplementary effect was noticed.

It appeared, however, that after inactivation at 65° C. the ability of the serum to fix complement in the presence of specific antigen was somewhat impaired. On the other hand, it seemed that a temperature of 62·5° C. had no appreciable adverse effect on the specific fixing capacity, and this temperature was therefore used for inactivating the sera for the test. The sera were diluted with equal parts 0·9 per cent. saline before inactivation to obviate the precipitation of the proteins of serum.

Haemolytic System.—Sheep erythrocytes and anti-sheep rabbit serum were used in the indicator system. The anti-sheep haemolytic rabbit serum was obtained by immunising rabbits with minced guinea-pig kidney. After dilution with equal parts of glycerol the titre of this amboceptor against sheep cells was about 1 : 10,000. The method was used because it was feared that an amboceptor containing antibodies against sheep cell stroma might react with normal sheep antigens present in the extracts of intestinal mucous membranes employed as antigen in the test. Such a combination might obscure the specificity of the reaction by fixing complement non-specifically.

* The buffered saline was made up as follows: $Na_2HPO_42H_2O$, 7·67 g.; KH_2PO_4, 2·27 g.; NaCl, 16·9 g. This was dissolved in 500 ml. of water and kept as stock solution. Diluted 1 : 4, it will give an isotonic solution with a stable *p*H.

The Technique of the Test

Before the main test was set up preliminary titrations were carried out each time to determine the titre of the complement both alone and in the presence of the antigen under examination and of at least one of the sera to be used. One tube in each series was used as control for a possible haemolytic effect of the antigen or the serum. The antigens were never found to exert any such effect. The " complement titre " used for any given test was that found in the titration series with the corresponding antigen. This titre should preferably not vary by more than half to one tube from that observed in the row " complement alone."

For the main test, dilutions of the serum to be used were prepared in volumes of 0·5 ml. All tubes except the first two in each row received 0·5 ml. saline. The first two tubes received 0·5 ml. of 1 : 1 dilution and the third tube 0·5 ml. of the serum dilution plus the 0·5 ml. saline. Half a ml. was then transferred from the third to the fourth tube and so on serially.

The amount of complement to be used for each test was calculated and mixed so that the appropriate amount (1½ MHD) for each tube should be contained in 0·25 ml. To this was then added the antigen contained in the same volume (0·25 ml.) of saline. All the tubes except the first in each row received 0·5 ml. of this mixture of antigen and complement. The first tube received the same amount of complement alone, diluted to 0·5 ml.

The mixture of serum, complement and antigen was incubated for 30 minutes at 37° C. A 3 per cent. suspension of washed sheep cells mixed with an equal volume of a dilution of amboceptor made up to contain 3 MHDs for each tube, constituted the haemolytic system. One-half ml. of this was added to each tube and the mixture incubated at 37° C. for 30 minutes. After the second incubation in the water bath the racks were placed in the refrigerator over night, and the reaction was read the following morning, when the cells had settled to the bottom.

It is very important to read the strength of the reaction quantitatively. A method of doing so has been described (Sigurdsson, 1945). It is indispensable, if the test is to be of reliable diagnostic value, that weakly positive sera of known strength be included in the test each time, and the strength of the reaction on any day be corrected, accordingly as the " control sera " give a reaction that is above or below their " standard value."

In this paper the strength of the reaction is expressed numerically according to a system previously described (Sigurdsson, 1945). Figures of less than 1·0 are not considered significant and up to 2·5 is called weakly positive.

Johnin tests

Two samples of PPD johnin (marked JP.35 of January 5th, 1942, and JP.4 of August 3rd, 1944, respectively) were kindly sent to us by

Mr. R. E. Glover, of the National Institute for Medical Research, Hampstead, England; for use in the tests. The materials were dissolved and are referred to as " English johnin " in the results summarised in Table I.

Further samples of johnin PPD were prepared in our laboratory from cultures of a bovine strain of *M. johnei* (ARC, 127) kindly sent to us by Mr. Glover. The method of preparation was that supplied by Dr. H. H. Green, of Weybridge (personal communication), which greatly simplifies the procedure of obtaining johnin PPD.* This product was dissolved and set up for testing in the same way as the " English johnin." In the first test (August 29th, 1944) the strength used was 4 mg./ml. (in terms of the dry weight of PPD) in the case of our johnin and 3 mg./ml. in the case of the " English johnin " and in the second test (September 27th) 3 mg./ml. in both cases. The two johnins were injected simultaneously into the skin of opposite limbs. Two successive injections of 0·1 c.c. in each case were made with an interval of 48 hours between them, and the skin reaction was recorded 24 hours after the second injection. From preliminary tests on another flock of sheep, heat-concentrated johnin seemed to be less satisfactory than the PPD, and it is therefore believed that it would not have given better results in the tests here reported.

At *post-mortem* examination the intestines were examined in some detail. In Table I a distinct and typical thickening of the intestinal mucosa is indicated by a ++ sign. A slight thickening of the intestinal mucosa or a typical change found only in a mesenteric gland is designated +. When a small portion of the intestinal mucosa, or when a mesenteric gland was considered suspicious, but no definite lesion was found, it is indicated by a ± sign. Four smear preparations were taken from each intestine, stained by the Ziehl-Neelsen method and examined microscopically.

RESULTS

In May, 1944, 55 sheep from an infected flock were brought to our experimental farm situated in the vicinity of Reykjavik. This flock had probably been free from Johne's disease until early in 1944, when the owner lost some sheep from this cause. The sera from these sheep were tested several times while the flock was kept at the experimental farm, and johnin tests were carried out twice. Late in September, 1944, the sheep were slaughtered and minutely examined *post mortem*, the findings being then correlated with the results of the *ante-mortem* tests.

* In this method the protein derivative is precipitated from the filtrate of the steamed culture with trichloracetic acid at a concentration of 4 per cent. washed centrifugally, first with diluted trichloracetic acid (1 per cent.) and then with water lightly buffered with KH_2PO_4 to remove the residual trichloracetic acid. The wet precipitate can either be brought to a dry form by washing, first with acetone and then several times with anhydrous ether, or alternatively, redissolved in alkaline buffered phosphate brought to the neutral point, and diluted to a standard protein concentration after determination of the N by microKjal.

TABLE I

COMPARATIVE RESULTS OF JOHNIN AND COMPLEMENT FIXATION TESTS (CFT) ON A FLOCK OF 55 SHEEP INFECTED WITH JOHNE'S DISEASE

Tests carried out between May 11th and September 29th, 1944. In the last two columns the *post-mortem* findings are shown. (Sixteen sheep were not submitted to the full johnin tests)

Sheep No.	CFT Titres (DS) May 11th	CFT Titres (DS) June 27th	CFT Titres (DS) Aug. 29th	Johnin Tests, Aug. 29th: English johnin	Johnin Tests, Aug. 29th: Icelandic johnin	CFT Titres (DS) Sept. 19th	Johnin Tests, Sept. 19th: English johnin	Johnin Tests, Sept. 19th: Icelandic johnin	CFT Titres (DS) Sept. 29th	P.M. Findings (Intestines): Macroscopic	P.M. Findings (Intestines): Microscopic
1	6	—	1	—	—	—	—	—	0·5	—	—
2	—	—	—	—	—	—	—	—	—	—	—
3	—	—	6	++	+	1	++	++	4	++	+++
4	—	—	0·5	—	—		—	—	—	—	—
5	—	—	0·5	—	—	2	—	—	1·5	—	+
6	—	5	4·5	++	++	2	+	+	1	++	+++
7	—	1	1	—	±	0·5	—	±	n.t.	++	—
8	2·5	7·5	*							++	++++
9	—	3·5	2	±	+	4·5	+	+	10	++	+
10	—	2	4·5	—	±	1	—	—	1·5	—	(+)
11	—	7	1	—	—	4·5	±	±	4·5	—	+
12	—	—	1	—	—		+	±	2·5	—	—
13	—	1·5	1·5	±	+	4	—	—	8·5	++	+
14	—	2		±	±	4·5	—	—	6	+	+
15	—	0·5	2	—	—	0·5	—	—	6	++	—
16	0·5	6·5	*							—	—
17	—	9·5	0·5	+	+	5	+	±	n.t.	+	+
18	—	—	—	—	—	—	—	—		—	—
19	—	2·5	2	—	±	n.t.	—	—	8·5	+	+
20	—	4	2	+	+	1	++	++	2·5	++	++
21	—	1	0·5	—	+	1	—	—	8	—	±
22	—	3·5	0·5	±	±	4	±	±	6	—	+
23	—	5·5	1	±	—	0·5	—	—	2·5	±	+
24	2	7	1·5	+	++	3	++	++	7·5	++	+++
25	2	4	*							++	++++
26	2	n.t.	*							++	+
27	—	—	*							—	—
28	4	4·5	*							++	++++
29	0·7	—	*							—	—
30	—	1	—	—	+	3	—	—	6·5	±	+
31	—	2·5	—	±	±	—	±	±	n.t.	—	—
32	—	3·5	*							—	—
33	—	—	*							—	—
34	—	2	*							—	—
35	—	4	*							—	+
36	—	—	0·5	—	—	—	—	—	—	—	—
37	—	10	1	+	++	6·5	±	±	9	+	—
38	—	—	*							—	—
39	—	—	*							—	—
41	—	5·5	*							++	+
42	—	6	*							—	++
43	8	3	9	—	++	8	—	++	10	++	++++
44	—	3	2·5	—	—	1·5	—	—	8	—	—
45	—	n.t.	0·5	—	—	—	—	—	n.t.	—	—
46	—	—	—	—	—	—	—	—	—	—	—
47	—	0·5	*							—	—
48	—	3	1	—	—	—	±	±	1·5	—	+
49	—	1		++	++	6	++	++	10	+	+
K1		5	10	±	++	10	++	++	10	++	++
K2			—	—	—	—	±	—	—	—	—
K3		3	—	—	—	0·5	—	—	4	++	+
K4			—	—	—	1·2	—	—	6·5	—	—
K5		6	++	++	6·5	++	++	9	++	++
K6			—	±	±	—	—	—	0·5	—	—
K7			0·5	±	++	?	±	++	9·5	—	+

* = Dead or killed before this date.
n.t. = Not tested.

Lesions of Johne's disease were found in 31 out of the 55 slaughtered sheep (Table I). All but one of the 31 which showed these lesions had given a positive complement fixation test shortly before they were killed. The one exception (No. 7) was a sheep whose blood sample had been lost in transit, and for that reason the test could not be performed. On one earlier occasion the serum from this sheep, however, had been weakly positive. In four other sheep that were serologically positive (Nos. 16, 21, 44 and K4), no signs of Johne's disease could be found *post mortem*. Two more sheep (Nos. 32 and 34) would seem also to fall into this category, although the sera taken from them at the time of slaughter did not fix complement.

The test carried out on sera taken at this time is not included in the table.

The results of the second (September 9th) johnin tests on the sheep, *i.e.*, those tests performed a short time before slaughter (late September, 1944) may be briefly summarised as follows :—

English johnin : 13 positive (++ and +) reactors. Two of these reactors showed no macroscopic lesions of Johne's disease *post mortem*.

Icelandic johnin : ten positive (++ and +) reactors. Lesions of Johne's disease were found in all these reactors.

In the table those cases in which we were uncertain whether or not the skin at the site of injection remained of entirely normal thickness (and hence a decisive positive reaction could not be recorded) are indicated by a ± sign. It is hardly possible to assess the significance of this type of reaction in the flock under study, where so many sheep were infected and therefore the random coincidence of ± and disease was bound to be high. Furthermore, it was almost a matter of chance in some cases whether the result of the test was reported as entirely negative or doubtful (±).

In the first johnin tests, begun on August 29th, 16 sheep reacted to the Icelandic johnin and seven to the English johnin. At that time, as already said, a concentration of 4 mg./ml. was used in the case of our johnin and only 3 mg./ml. in the case of the English preparation. In the second test, however, both preparations were used in the same concentration, *i.e.*, 3 mg./ml.

Correlation between the Complement Fixation Test and the Johnin Test

Sixteen sheep were killed before the johnin tests were begun, and therefore the johnin tested cases are somewhat fewer than those serologically tested. The following figures are extracted from the table to summarise this correlation :—

First series (johnin tests and complement fixation tests carried out at the same time)

Of 20 serologically positive sheep, 12 reacted to our Icelandic johnin.

Of 20 serologically positive sheep, 7 reacted to English johnin.

Of 16 reactors to our Icelandic johnin, 12 were serologically positive.

Of 16 reactors to our Icelandic johnin, 4 were serologically negative.

Of 7 reactors to English johnin, 7 were serologically positive.

Of 7 reactors to English johnin, 7 reacted to our Icelandic johnin.

Of 6 doubtful (±) with our Icelandic johnin, 5 were serologically positive.

Of 10 doubtful (±) with English johnin, 7 were serologically positive.

Second series (*johnin tests and complement fixation tests carried out at the same time*)

Of 21 serologically positive sheep, 10 reacted to our Icelandic johnin.

Of 21 serologically positive sheep, 10 reacted to English johnin.

Of 10 reactors to our Icelandic johnin, 10 were serologically positive.

Of 10 reactors to our Icelandic johnin, 0 was serologically negative.

Of 13 reactors to English johnin, 10 were serologically positive.

Of 13 reactors to English johnin, 3 were serologically negative.

Of 9 doubtful cases (±) with our johnin, 4 were serologically positive.

Of 4 doubtful (±) with English johnin, 2 were serologically positive.

Johne's disease of sheep was only recently introduced into Iceland, and it is still limited to certain districts. It is therefore possible at present to obtain blood from sheep that are certainly free of the disease to perform control tests. Thus, tests upon 118 blood samples received from three different non-infected areas revealed four only that were weakly positive (DSs 2, 1, 3, 1, respectively—see Sigurdsson, 1945). Stronger reactions could therefore be justifiably assumed usually to indicate infection.

DISCUSSION

The complement fixation test (CFT) as previously described by us, using an antigen extracted from infected intestinal mucosa, was found to be a satisfactory diagnostic test for Johne's disease of sheep. In the present study it proved to be superior to the intradermal johnin test in that the latter failed to detect a large proportion of the infected animals.

With one possible exception (No. 7), the CFT succeeded in detecting all infected sheep. Four serologically positive sheep showed no lesions of Johne's disease *post mortem* (Nos. 16, 21, 44 and K4, with DSs of 6·5, 8·0, 8·0 and 6·5 respectively). In evaluating these results it should be borne in mind that no such strong reactions have been encountered in healthy flocks, and that it is extremely difficult, and even impossible, definitely to exclude the possibility of infection in a sheep from an infected flock. If no macroscopic lesions are to be found to indicate the exact site of infection, the bacteria may be very hard to find. It seems, therefore, quite possible that the above four sheep were also infected, although no morphological signs were found to indicate this.

In three cases (Nos. 7, 15, and 37) the intestines were typically thickened, but no bacteria could be detected microscopically in the lesions. This curious finding, that in some cases of typical Johne's disease no acid-fast bacteria can be found, is in agreement with the observations of other workers and earlier experience in this laboratory.

By the date of presentation of this paper about 10,000 sera from infected farms had been subjected to the complement fixation test. It is evidently impossible to assess accurately the reliability of such tests in the course of work carried out in the field. It may be said, however, that the results very definitely tend to substantiate the conclusion, that the present CFT is a fairly reliable diagnostic test for Johne's disease of sheep. The need to employ the complement fixation test in a standardised quantitative form must, however, be stressed.

We have tested a number of sera from active human cases of tuberculosis and leprosy with this new antigen (unpublished work) and found most of them capable of fixing complement in the presence of this antigen, many even in high dilutions. The reaction with this antigen is therefore not specific for infections with *M. johnei*. It is also applicable to infections with other members of the acid-fast group. This should be kept in mind in attempts to use it for diagnostic work upon cattle.

With regard to the question of false positive serological reactions and the problem of latent infections in general, the following history of the recent origin of Johne's disease in Iceland is interesting to note. In the year 1933, sheep of the Karakul breed were imported into Iceland from Germany, having been purchased from the "*Institut für Tierzucht und Molkereiwesen an der Universität, Halle*" and believed to be in good health. On arrival they were placed in quarantine for some time and then distributed to 16 different farms scattered all over the country. Each farmer received one ram, except in one case where five animals, some of them ewes, were retained together. Five years later Johne's disease was discovered for the first time in Iceland on one of these farms, and at about the same time infection was found also to have taken place on five of the

16 farms mentioned above into which the rams had been introduced (Gíslason, 1939). The disease is still limited to areas around the original foci of infection, but it has caused heavy losses in all the infected districts. This seems to afford a conclusive proof, that at least five of the 20 original Karakul sheep were harbouring a latent infection with *M. johnei*, in so far as they were able to establish enzootic foci, when introduced into a susceptible population. As is well known, Johne's disease of sheep is not considered to be prevalent in Germany.

It may also be remarked, parenthetically, for it has no direct bearing on the subject of the present paper, that, besides Johne's disease, two serious lung infections of sheep were introduced with this same flock of Karakul sheep.

SUMMARY

The results of a new diagnostic complement fixation test for Johne's disease among sheep are described. The antigen used was extracted from the infected intestinal mucosa. The study was carried out on an infected flock of 55 sheep. Out of 31 sheep found to be infected on slaughter, 30 had proved to be serologically positive, the remaining one not having been subjected to an adequate test. The sera from four more sheep in which no lesions could be found in the intestines, were also found to be strongly positive.

One hundred and eighteen sheep from healthy areas were tested as controls. Four only of them showed serological reactions, which were very weak.

Thirty-nine sheep of the experimental flock were also tested by means of the intradermal johnin test, using johnin PPD as described. Only about 50 per cent. of the infected sheep reacted to the various johnins used in the strengths mentioned in this paper.

REFERENCES

Dunkin, G. W. (1928). *J. comp. Path.*, **41**, 94.
Glover, R. E. (1941). *Vet. J.*, **97**, 179.
Hagan, W. A., and Zeissig, A. (1933a). *J. Amer. vet. med. Assoc.*, **82**, 391; (1933b). *Cornell Vet.*, **23**, 1.
M'Fadyean, J., Sheather, A. L., and Edwards, J. T. (1916). *J. comp. Path.*, **29**, 201.
Sigurdsson, B. (1945). *J. Immunol.* (in press).
Gíslason, G. (1939). *Um garnaveiki í sauofé* (in Icelandic). A pamphlet, 17 pp.

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THE RELATIONSHIP OF DOSAGE AND SITE OF INOCULATION TO THE AGGLUTININ RESPONSE TO *BRUCELLA ABORTUS* STRAIN 19 VACCINE: A COMPARISON OF THE SUBCUTANEOUS, INTRACUTANEOUS AND INTRACAUDAL ROUTES

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INTRODUCTION

The Bureau of Animal Industry of the U.S. Department of Agriculture prescribes that calves to be immunised against contagious abortion by means of strain 19 vaccine shall be injected subcutaneously with a dose of 5·0 ml. of vaccine containing from 8×10^9 to 12×10^9 living cells per ml. Vaccinated animals quickly become reactors to the *Br. abortus* agglutination test, but it is claimed that whereas about 95 per cent. of calves vaccinated at four to eight months of age become negative to the test 12 months after vaccination, a high proportion of adult animals may remain positive for very long periods. Since agglutination reactions due to vaccination with strain 19 cannot be differentiated from those due to infection with virulent strains, reacting cattle must be excluded from milk production in many parts of the U.S.A., and furthermore they interfere seriously with the application of the test-and-slaughter method of eradicating brucellosis. For these reasons the B.A.I. has recommended that strain 19 vaccination be restricted in general to calves between four and eight months of age (the so-called calfhood vaccination) and applied to adults under certain conditions only. However, for reasons recently discussed by Turner (1944) the scheme accepted by the Australian Agricultural Council and initiated by the Victorian Department of Agriculture last year, embraced not only calves but adult cows. Although no reference to untoward reactions had then appeared in the literature, it was found that in some herds up to half of the adult animals developed excessive local inflammatory reactions with considerable systemic disturbance and diminution or even cessation of milk yield. The local reaction persisted for many months and thus constituted a very undesirable blemish, especially in stud animals. In addition, the relatively large doses, 5·0 ml., introduced difficulties of manufacture, transport and cost.

For these reasons, it was considered desirable to determine whether, without impairing the antigenic response as judged by the agglutination test, (*a*) the dosage might be reduced, (*b*) a site and route of inoculation might be chosen which would lead to less local reaction and systemic disturbance, and (*c*) the duration of a positive agglutination reaction might be shortened. The agglutination reaction was used as a measure of the immune response on the assumption that any procedure which affects the response to the antigens concerned in agglutination would similarly affect the

response to the antigens concerned in resistance. We hope to obtain direct evidence of the validity of this assumption in subsequent experiments in which some of the cows used in this investigation will be tested for resistance to infection with virulent *Br. abortus*.

The decision to investigate sites of inoculation other than the subcutaneous was stimulated by a report from the University of Maryland Experiment Station (Corbett, 1941-42), which stated that after intracutaneous (= intradermal) inoculation of calves with 0·1 ml. of vaccine there was "no difference in the degree of immunity by the subcutaneous and intradermal methods." It also stated that both calves and adults inoculated by the intracutaneous method lost their positive agglutination reactions sooner than similar animals inoculated by the subcutaneous method. Crawford (1944) referring to the same investigation, stated that the milk yield of cows inoculated with 0·1 ml. or 0·2 ml. of vaccine by the intracutaneous method was not impaired after inoculation. We therefore included intracutaneous inoculation in our experiments. The subcutaneous fibrous tissue at the tip of the tail is used exclusively as the site of introduction of bovine pleuro-pneumonia vaccine in Australia, where hundreds of thousands of cattle are vaccinated each year; it was therefore decided to determine whether the tip of the tail might be a suitable site for introducing strain 19 vaccine. The advantages of this site in pleuro-pneumonia vaccination include the ease of injection of semi-wild animals, the fortunate tendency for the resulting infection to remain localised and not to invade the body tissues generally, and the relative unimportance of the caudal extremity if serious reactions should occur.

METHODS AND MATERIALS

The samples of vaccine were freshly prepared; they were of standard viability (8×10^9 to 12×10^9 viable cells per ml.) when used and the cells were entirely in the smooth phase. The vaccine was prepared in accordance with instructions issued by the United States Bureau of Animal Industry. In those experiments in which a dose of 0·04 ml. of vaccine was to be used, a 1 : 5 dilution of standard vaccine in the prescribed sterile buffered saline was prepared immediately before use, and 0·2 ml. was injected.

The cows used in the first two experiments were grade dairy Shorthorn cows (non-reactors for *Br. abortus*) which had completed their third lactation and were pregnant when inoculated, some of the cows in the second experiment being well advanced in pregnancy (up to eight and a half months). All animals, before injection, were negative to the agglutination test by the usual standards. Agglutination tests on serum samples collected before inoculation showed that 33 did not reach a titre of 20, 66 had a titre of 20, and eight had a titre of 40. The cows used in the third experiment were grade dairy Shorthorn heifers, aged 15 to 18 months. Pre-injectional agglutination tests showed that the serum of 28 of the heifers did not reach a titre of 20, five had a titre of 20, two had a titre of 40, and one had a titre of 80.

In the intracaudally inoculated animals the vaccine was injected deeply into the fibrous tissue about ½ inch from the tip, with the aid of a gauge 18 needle 1 inch long. The site of inoculation for the animals inoculated subcutaneously and intracutaneously was over the seventh or eighth rib on a level with the acromion. For convenience we refer in this communication to inoculation into the subcutaneous fibrous tissue at the tip of the tail as intracaudal inoculation, to inoculation with 0·2 ml. of vaccine intracutaneously as intracutaneous inoculation, and to inoculation with 5 ml. of vaccine subcutaneously as the standard procedure.

Since the minimal infecting dose of strain 19 for guinea-pigs has been shown to be of the order of 470×10^3 cells (Hutchings, 1943), a cultural method, adapted from that described by Huddleson (1943), was used for the examination of milk and colostrum samples for the presence of the strain 19 organism. The samples were stored overnight in the cold to allow the cream to rise by gravity. Three drops (about 0·15 ml.) of the cream were spread over the surface of potato-serum-agar plates containing 1 : 700,000 crystal violet. The plates were incubated without added CO_2 for four to six days at 37° C., because the strain 19 organism grows freely in air. Tests showed that when approximately 1,000 viable strain 19 cells per ml. were added to a sample of milk which itself contained no *Br. abortus* by the cultural test, typical *Br. abortus* colonies grew up on the plates sown from it in the expected numbers (*c.* 100–150 per plate).

As antigen for the agglutination test we used a suspension of strain 19 cells, of density equivalent to the No. 2 tube of Brown's opacity standards, in 0·85 per cent. saline with 0·25 per cent. of phenol added as preservative. To a series of doubling dilutions of serum, starting with a basal dilution of 1 : 10, equal volumes of antigen were added. The serum-antigen mixtures were incubated at 37° C. for 24 hours and the results then read. The reciprocal of the dilution of serum in the last tube in which complete or partial agglutination had occurred was taken as the agglutination titre.

EXPERIMENTAL

In the first experiment 60 pregnant grade dairy Shorthorn cows which had completed their third lactation period were divided into six groups of ten animals. Groups 1–4 were injected subcutaneously with 5·0 ml., 1·0 ml., 0·2 ml. and 0·04 ml. of vaccine respectively. Group 5 was injected with 1·0 ml. intracaudally. Group 6 was injected with 0·2 ml. intracutaneously.

A second experiment was undertaken with slightly larger groups ; 47 cows, similar to those used in the first experiment, were divided into two groups of 16, which received respectively 5·0 ml. and 1·0 ml. of vaccine subcutaneously, and a third group of 15 which received 1·0 ml. of vaccine intracaudally. In addition a private herd of 20 lactating cows, nine of which were reactors for *Br. abortus*, was injected intracaudally with 1·0 ml. of vaccine in order to obtain observations upon milk yield. These animals were not included in the statistical treatment.

In the third experiment the effect of reduced dosage by the intracaudal method of inoculation was investigated. Three groups of 12 grade dairy Shorthorn heifers, the majority of which were

non-pregnant, were injected intracaudally with 1·0 ml., 0·2 ml. and 0·04 ml. of vaccine respectively.

Local Reactions

The local reactions resulting from the different methods are summarised as follows :—

Subcutaneous inoculation.—Local reactions were in the form of firm, sometimes painful inflammatory " plaques," adherent to the underlying tissue, and ranging in size, according to dosage, from 3 to 6 inches in diameter with the 5 ml. dose to 1 to 2 inches in diameter with the 0·04 ml. dose when examined four days after inoculation. The reactions gradually subsided ; two to three weeks after inoculation they were about 1 to 2½ inches in diameter with the 5 ml. dose and had almost completely disappeared with the lower doses.

Intracaudal inoculation.—Reactions in the animals inoculated intracaudally when examined four days after inoculation were in the form of cylindrical swellings of the terminal 2 to 4 inches of the tail, the swelling being in general larger with the larger dose. In about 20 per cent. of the animals inoculated with 1·0 ml. the swelling was accompanied by a slight serous exudation. The swellings soon subsided and the tip of the tail became hard and brittle. Two to three weeks after inoculation the terminal 1 to 1½ inches was found to have sloughed off and healed.

Intracutaneous inoculation.—The local reactions were confined to the skin, and four days after inoculation were in the form of raised swellings ¾ to 3 inches in diameter ; in some cases a necrotic centre sloughed out. A week after inoculation the reactions had almost disappeared and two weeks after inoculation were undetectable.

Milk Yield

Observations on the milk yield were confined to the dairy herd inoculated intracaudally with 1·0 ml. The milk yield fell by about 40 per cent. during the 48 hours after inoculation, began to rise again on the third day and by the sixth day after inoculation had returned to the normal level. The mean loss of milk per cow over the period was 35 lb., representing a 28 per cent. loss.

Calvings

Most of the 107 cows used in the first two experiments were pregnant when inoculated, many of them being well advanced in pregnancy.

Ninety of these have now calved. Of these, 89 cows carried their calves to full term and calved normally. One cow, which had received 1·0 ml. intracaudally six days earlier, gave birth to a weak living calf (at eight and a half months) and had retained membranes, but cultural examination of swabs from the uterus, material from the placenta, and of milk, failed to detect the presence of *Br. abortus*.

TABLE I

THE GEOMETRIC MEANS, STANDARD DEVIATIONS AND RANGES OF THE MAXIMAL AGGLUTINATION TITRES OF THE COMBINED GROUPS, COMPARED WITH THE STANDARD TREATMENT

Treatment	*No. of Animals*	*Range of Titres*	*Mean Titre (Geometric)*	*Relative Mean Titre (Standard Treatment = 100)*	*Mean (Log_{10})*	*S.D.*	*Significance of the Differences from the Standard Procedure*	
							Probability	*Significance*
1·0 ml. intracaudal ...	37	2,560–83,920	11,890	220	4·08	0·33	<0·001	HS
0·2 ml. intracaudal ...	12	5,120–20,480	7,670	142	3·88	0·27	0·2–0·1	NS
0·04 ml. intracaudal...	12	2,560–40,960	7,240	134	3·85	0·33	0·3–0·2	NS
5·0 ml. subcutaneous	26	320–20,480	5,400	100	3·73	0·38	—	—
0·2 ml. intracutaneous	9	1,280–10,240	3,760	70	3·68	0·28	0·7–0·6	NS
1·0 ml. subcutaneous	26	320–10,240	3,250	60	3·51	0·17	0·02–0·01	S
0·2 ml. subcutaneous	10	640–10,240	2,230	41	3·35	0·34	0·01–0·001	HS
0·04 ml. subcutaneous	9	640–5,120	2,030	38	3·31	0·36	0·01–0·001	HS

NS = Not significant at 5 per cent. level. HS = Significant at 1 per cent. level. S = Significant at 5 per cent. level.

Localisation of Infection in the Udder

There is a suggestion from the work of Cotton, Buck and Smith (1933) that the intracutaneous route might favour localisation in the udder. During the period from one week to two months after inoculation we examined 52 samples of milk and colostrum from cows injected with 1·0 ml. of vaccine intracaudally or with 5·0 or 1·0 ml. subcutaneously, but consistently failed to isolate strain 19.

Agglutination Reactions.

The results are presented graphically in Figs. 1 to 3, and in Table I.

In Figs. 1 to 3 respectively the means of the logarithms of the titres in each group at each bleeding are plotted against the time after inoculation in days. For convenience the scale for the mean titres is also shown on the right-hand ordinate. It is strikingly evident in all three experiments that the maximal response was consistently obtained with 1·0 ml. intracaudally. In each group, irrespective of the route of inoculation or the dosage, the maximal agglutination titre was obtained at about 14 days after inoculation, although a few animals reached their maximal titres a little later. The only exception was the group inoculated with 1·0 ml. subcutaneously in experiment I (Fig. 1), in which the mean titre was very slightly higher 22 days after inoculation. One month after inoculation the titres declined to less than one-half the maximal value at 14 days after inoculation.

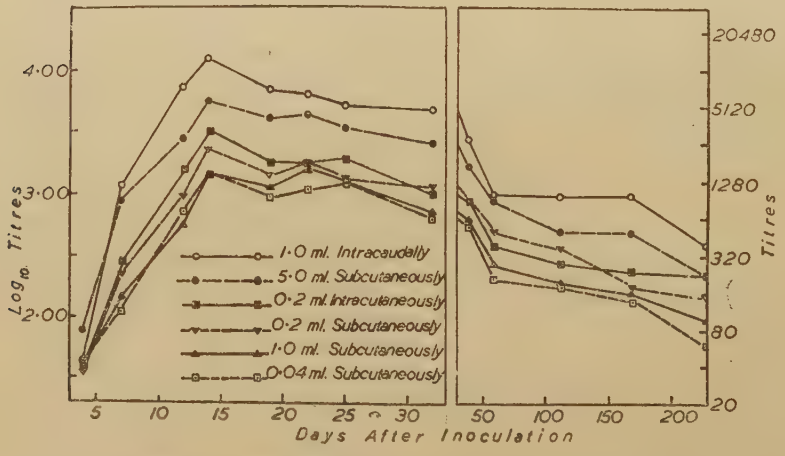

FIG. 1. The agglutinin responses of adult cows to inoculation with *Br. abortus* Strain 19 vaccine as follows : 1·0 ml. intracaudally ; 5·0 ml., 1·0 ml., 0·2 ml., and 0·04 ml. subcutaneously ; and 0·2 ml. intracutaneously.

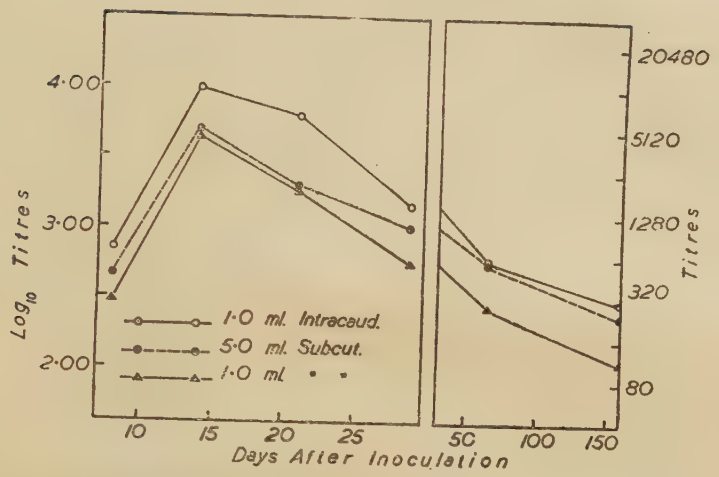

FIG. 2. The agglutinin responses of adult cows to inoculation with *Br. abortus* Strain 19 vaccine as follows: 1·0 ml. intracaudally; 5·0 ml. and 1·0 ml. subcutaneously.

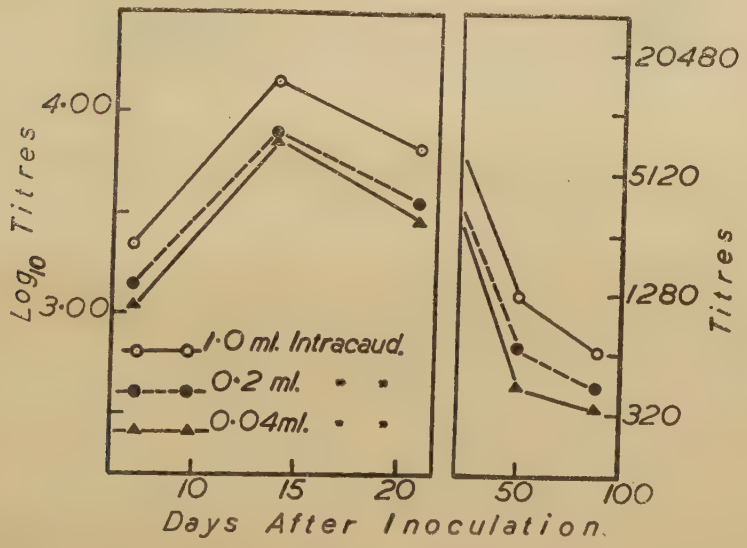

FIG. 3. The agglutinin responses of adult cows to inoculation with *Br. abortus* Strain 19 vaccine as follows: 1·0 ml., 0·2 ml. and 0·04 ml. intracaudally.

STATISTICAL ANALYSIS OF RESULTS

The figures obtained in the three experiments at the 14 to 15 day bleedings, when the titres of the groups were at a maximum, were examined statistically. The range of titres of animals subjected to the same treatment was very wide (see Table I). Frequency diagrams showed that the titres were very skewly distributed and that the arithmetic mean was far removed from the mode. On the other hand, the logarithms of titres were more

or less symmetrically distributed about the mean of the logarithms; the statistical computations were therefore carried out with the logarithms of titres and the geometric mean was taken as a more accurate estimate of the mean response. The agglutination responses to inoculation with living, and therefore reproducible, *Br. abortus*, thus behave like antitoxic titres to staphylococcal toxoid (Smith, 1936) and to *Cl. welchii* type D epsilon toxoid (Turner & Rodwell, 1943).

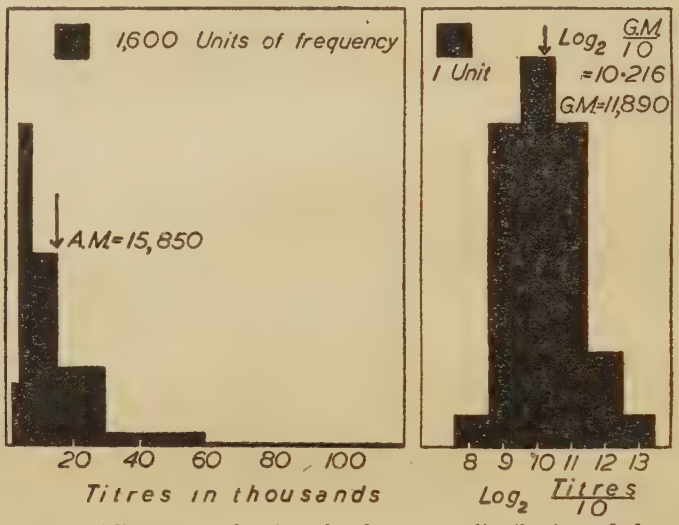

FIG. 4. Histograms showing the frequency distribution of the maximal agglutinin titres of 37 cows inoculated with 1·0 ml. intracaudally.
Left: abscissae in arithmetic units. Right: abscissae in logarithmic units.

Advantage was taken of the doubling series of dilutions to simplify the calculations. Instead of using the $\log_{10}$ of the titres, the titres were divided by 10 and then expressed as $\log_2$. This procedure results in small whole numbers on a logarithmic scale. Thus a titre of 20 becomes 1, 40 becomes 2, and so on. The results of the calculations may easily be converted to $\log_{10}$ if desirable by multiplying by 0·30103 (*i.e.*, $\log_{10}2$) and since one-tenth of the titres were taken, adding 1 to the characteristic.

An analysis of variance was first carried out by comparing the three treatments common to the first two experiments, *i.e.*, 50 ml. and 1·0 ml. subcutaneously, and 1·0 ml. intracaudally. It was found that the "between experiments variance," *i.e.*, the variance due to the different batches of vaccine used, was quite insignificant. Those groups which received the treatment common to all the experiments, *i.e.*, inoculation with 1·0 ml. intracaudally, were next compared and the variances were found also to be quite insignificant. This was considered sufficient justification for combining all the animals in the three experiments which had received the same treatment. Two animals responded exceptionally poorly; one which received 0·04 ml. subcutaneously and one which received 0·2 ml. intracutaneously gave maximal titres of only 80 and 320 respectively. These titres differed from the mean of the remainder in the respective groups by more than three times the standard deviation of their group; they were therefore excluded from the analysis.

In Table I are included the total numbers of animals inoculated by each method, the range of the titres obtained by each treatment 14 days after inoculation, the means of the logarithms of these titres and their standard deviations, and the geometric means (the antilogarithms of the mean logarithms). Also included are the mean responses to each treatment expressed as a percentage of the mean response to the "standard" treatment, together with the statistical significance of the differences.

The results of the analysis of variance are shown in Table II.* The value for F is very highly significant and indicates that significant differences exist between the means of some of the groups.

TABLE II

ANALYSIS OF VARIANCE OF COMBINED GROUPS

Source of Variance	*Sum of Squares*	*Degrees of Freedom*	*Variance*	*F*
Within group ...	142·7065	133	1·0730	13·5
Between group ...	100·9953	7	14·4279	

The "t" test was therefore applied to the groups in pairs, and the results are set out in Table III, in which the groups are arranged in descending order of their means.

TABLE III

SUMMARY OF RESULTS OF THE "t" TEST: TREATMENTS ARRANGED IN DESCENDING ORDER OF THEIR MEANS

	0·2 *ml. Intra-caudal*	0·04 *ml. Intra caudal*	5·0 *ml. Sub-cutaneous*	0·2 *ml. Intra-cutaneous*	1·0 *ml. Sub-cutaneous*	0·2 *ml. Sub-cutaneous*	0·04 ml. *Sub-cutaneous*
1·0 ml. intra-caudal ...	NS	S	HS	HS	HS	HS	HS
0·2 ml. intra-caudal ...	—	NS	NS	NS	HS	HS	HS
0·04 ml. intracaudal..	—	—	NS	NS	HS	HS	HS
5·0 ml. sub-cutaneous.	—	—	—	NS	S	HS	HS
0·2 ml. intra-cutaneous.	—	—	—	—	NS	S	HS
1·0 ml. sub-cutaneous.	—	—	—	—	—	NS	NS
0·2 ml. sub-cutaneous.	—	—	—	—	—	—	NS

Thus, reading along the top line of the table it is seen that the mean of the titres of the animals inoculated intracaudally with 1·0 ml. was not significantly greater than that of the animals inoculated with 0·2 ml. intra-

* The values for the sums of squares and variances are in the $\log_2$ titre/$_{10}$ notation.

caudally, but was significantly greater (at the 5 per cent. level) than that of the animals inoculated with 0·04 ml. intracaudally and was highly significantly greater (at the 1 per cent. level) than the means of the titres of the animals inoculated with various doses by the other methods.

Since all degrees of response to a given treatment, from maximum to virtually zero, may be expected in a population of normal animals, the two animals, injected with 0·2 ml. intracutaneously and 0·04 ml. subcutaneously, which responded very poorly cannot necessarily be regarded as being abnormal; they are probably only rare examples of a normal occurrence, which is in no way related to the particular treatments involved. Since the inclusion of such rare animals would place the treatment in which they occur at a disadvantage we considered it better to exclude them. If, however, the view be taken that the poor response of these animals may be related to the particular treatment, they must then be included. The value for the variance now becomes greater and the means of the groups receiving these treatments become lower, so that the differences between the means of the 1·0 ml. and the 0·04 ml. intracaudal treatments, and that between the 0·02 ml intracutaneous and 0·2 ml. subcutaneous treatments are no longer significant; while the differences between the means of the 0·2 ml. intracaudal and the 0·2 ml. intracutaneous treatments, and that between the 1·0 ml. subcutaneous and the 0·04 ml. subcutaneous treatments now become significant (P = 0·05), because of the lower values for the means.

The results of the " t " test (Table II) do not of course mean that the effects of the treatments between which no significance was demonstrated are necessarily identical: furthermore, the smooth trends of the mean titres in Fig. 5, which relates dosage with mean responses for each route of inoculation, strongly suggest that even with the largest doses investigated the maximal possible responses were not reached, and that dosage could have been reduced further before reaching the threshold, since the curves show no tendency to flatten towards the x-axis or y-axis respectively.

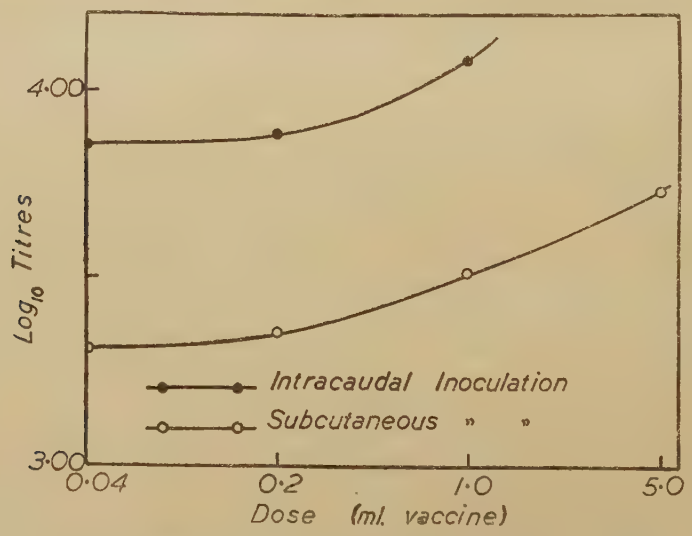

FIG. 5. The relationship of dosage to agglutinin response. Dosage by the intracaudal and subcutaneous routes respectively plotted against the logarithms of the maximal mean titres of the groups.

Discussion

The wide variation in response to identical treatments (Table I) emphasises how necessary it is to assess results of experiments of this nature on a statistical basis. Even though care is taken to select animals similar in age, breed and other characteristics, the overlapping of the ranges of titres indicates that unless groups are sufficiently large, comparatively large differences in the mean titres may have no significance. All the animals used in the comparison of agglutinin responses would be classed as negative to the agglutination test for brucellosis before inoculation; 42·7 per cent. had titres less than 20, 49·7 per cent. had a titre of 20, 7 per cent. a titre of 40 and 0·7 per cent. (one animal) a titre of 80. No relationship was observed between initial titre and subsequent agglutinin response.

It is clear from these experiments that the agglutinin response to the standard procedure (5·0 ml. subcutaneously) is significantly greater than that to 1·0 ml. subcutaneously and therefore that the dosage by this route cannot be greatly reduced without affecting the serological response.

The tissue into which the vaccine is introduced obviously is of great importance; the response to 0·2 ml. given either by the intracutaneous or intracaudal route is greater than that to the same dose given subcutaneously.

There is no doubt that in adult cows inoculation with 1·0 ml. intracaudally is greatly superior to either the standard procedure or to 0·2 ml. intracutaneously so far as agglutinin response is concerned. The milk loss after 1·0 ml. intracaudally is of some importance, but unfortunately we were unable to compare this with milk loss after the standard procedure or after intracutaneous inoculation. It is well recognised abroad and has been observed in the field in Victoria that this loss with the standard procedure may be very considerable, but the Maryland investigators claim that it is absent after intracutaneous inoculation (Crawford, 1944).

Local reactions to the standard procedure are never important in calves but may persist for long periods in adults. The local reaction to intracaudal inoculation in adults with 1·0 ml. may be classed as unimportant and is even less when 0·2 ml. is used. Our experience with intracutaneous inoculation supports overseas claims that local reactions in adults are unimportant and transient.

Apart from the transient effect upon milk yield, the advantages of intracaudal inoculation with 1·0 ml. over the standard procedure in adults are (*a*) a greater serological response, and therefore probably a greater immune response; (*b*) the unimportance of the local reaction; (*c*) economy of vaccine; and (*d*) the ease with which the injection can be made. This last is particularly important with semi-wild cattle if good crushes and ample assistance are not available, and would probably be even more important when handling calves. Since 0·2 ml. or even 0·04 ml. intracaudally gives at least

as good an agglutinin response as 0·2 ml. intracutaneously, is just as economical of vaccine, and has considerable technical advantages over intracutaneous inoculation, it only remains to determine whether it has any important effects upon lactation.

As to the duration of the positive agglutination status, since the rate of decline with all the treatments so far appears to be approximately the same, it is only to be expected that the better the serological response, the longer the period necessary for return to a negative status. There was no significant difference between the maximal titres with the standard procedure, and with 0·2 ml. intracutaneously or intracaudally; it is therefore to be expected that there would be little difference in the period necessary for return to a negative status. It appears from the Maryland Station Report (Corbett, 1941–42), that the grounds for stating that intracutaneous inoculation with 0·1 ml. of vaccine gave as good an immunity as the standard procedure were that the proportion of animals inoculated by the intracutaneous route which became positive to the agglutination test was as high as among the animals inoculated by the standard procedure, *i.e.*, they developed titres above an arbitrary level, probably 100. If this interpretation is correct, the maximal titres may still have been considerably less than those of the animals inoculated by the standard procedure, and it would then be expected that the titres of the intracutaneously inoculated animals would fall below the arbitrary level sooner than those inoculated by the standard procedure.

It is reassuring to find that since strain 19 was not found in any of the milk samples examined, there is no evidence that intracaudal inoculation favours localisation in the udder. It is also reassuring that all calvings in vaccinated pregnant animals have been normal, with one exception in which strain 19 could not be incriminated.

It will be observed that in all cases the agglutinin response to vaccine injected intracaudally and to 0·2 ml. intracutaneously was significantly greater than to the same dose injected subcutaneously. At present we are unable to offer any convincing explanation for this phenomenon.

It is proposed in future experiments to compare the value of the standard procedure, and of inoculation with 0·2 ml. intracutaneously and intracaudally in calves.

SUMMARY

In order to determine the influence of dosage and route of inoculation upon the agglutinin response to *Br. abortus* strain 19 vaccine, groups of adult cattle were inoculated by one of the following procedures: subcutaneously (5·0 ml., 1·0 ml., 0·2 ml. or 0·04 ml.), intracutaneously (0·2 ml.) or intracaudally (1·0 ml., 0·2 ml. or 0·04 ml.)—and maximal mean titres 14 days after inoculation were compared statistically.

The agglutinin response is closely related to dosage and to route of inoculation. The most efficient route for agglutinin production is the intracaudal, which in addition has technical advantages, and is free from severe and persistent local reactions. One ml. intracaudally is much more efficient than 5·0 ml. subcutaneously, is more economical, and causes a transient diminution of milk yield at least no greater than that reported after 5·0 ml. subcutaneously. Doses of 0·2 ml. or even 0·04 ml. intracaudally appear to be at least as efficient as 5·0 ml. subcutaneously or 0·2 ml. intracutaneously, but their effect upon milk yield has not been examined.

In our experience the greater the agglutinin response the longer the period necessary for return to negative agglutinin status.

Local reactions after subcutaneous inoculation were frequently considerable but after intracutaneous or intracaudal inoculation they were unimportant and transient.

Of 90 cows vaccinated during pregnancy 89 calved normally.

Strain 19 was not found in the mammary secretion of cows vaccinated subcutaneously or intracaudally.

ACKNOWLEDGEMENTS

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REFERENCES

Corbett, R. B. (1941–42). *Rep. Md agric. Exp. Sta.*, **55,** 18.

Cotton, W. E., Buck, J. M., and Smith, H. E. (1933). *J. agric. Res.*, **46,** 291.

Crawford, A. B. (1944). *Vet. J.*, **100,** 200.

Huddleson, I. F. (1943). *Brucellosis in Man and Animals*, p. 30. New York: The Commonwealth Fund [Revised Edit.].

Hutchings, L. M. (1943). *Michigan Sta. tech. Bull.*, **182,** 64.

Turner, A. W. (1944). *Aust. vet. J.*, **20,** 311.

Turner, A. W., and Rodwell, A. W. (1943). *Aust. J. exp. Biol. med. Sci.*, **21,** 27.

Smith, Margaret L. (1936). *J. Path. Bact.*, **42,** 227.

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EXPERIMENTAL ZINC PHOSPHIDE POISONING IN FOWLS

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INTRODUCTION

Although zinc phosphide (Zn_3P_2) has occasionally been sold as rat poison for at least 15 years (A. D. Campbell, personal communication), it was not used extensively in this country until war conditions interfered with the supply of Red Squill. Pest Officers of the Department of Agriculture for Scotland and War Agricultural Executive Committees in England have recently used a bait containing 5 per cent. zinc phosphide and as deaths amongst animals on premises where bait has been laid are frequently attributed to poisoning, this College in the last year or so has received many requests to examine viscera, especially of poultry, for evidence of zinc phosphide poisoning.

No information as to the symptoms and lesions produced by this material was to be found in the literature, though Mr. C. V. Watkins, M.R.C.V.S., kindly gave us an account of his experiences of the condition. Whilst methods for the quantitative determination of zinc were available, nothing was known about the quantities which might be expected in cases of poisoning, or the extent to which small traces found in visceral contents might be derived from the galvanised linings of food and water containers. There was, moreover, no very reliable method for the estimation of very small quantities of phosphide, and further it seemed possible that this substance might be rapidly oxidised in the alimentary tract and so escape detection. Thus, when asked by the Department of Agriculture to pronounce an opinion on fowls suspected to have died from zinc phosphide poisoning, we were rather diffident about cases in which analytical results were negative. Therefore, it was decided to investigate zinc phosphide poisoning in poultry to find the minimum lethal dose of the drug, and the degree of accuracy in diagnosis to be obtained from chemical tests.

Since this investigation began, Hare and Orr (1945) have briefly recorded one case of poisoning in geese and two cases in fowls in which traces of zinc phosphide were found in the crop and gizzard contents, but have given no details as to the total amounts recovered or the methods of estimation used. Later, Ingram (1945) described in considerable detail a case of poisoning in a colt, which was found to have over half a gramme of zinc phosphide in its stomach, but he, too, gave no information as to the analytical methods employed.

METHODS

Small gelatine capsules containing varying doses of a mixture of 10 per cent. zinc phosphide and 90 per cent. starch were given by

mouth to adult fowls. After death the alimentary tract was removed intact and ligatures tied at each end of the crop, gizzard and intestine; in certain cases other tissues such as liver, kidney, heart, lung and pectoral muscle were also examined.

Phosphide estimations were made as soon as possible on the contents of crop, gizzard and intestine using a modification of the method of Elmore and Roth (1943) which allows of the colorimetric determination of small quantities of volatile phosphorus. A weighed amount of the material to be examined was placed with 50 ml. distilled water in a 150 ml. flask fitted with a stopper carrying a small separating funnel, an air inlet-tube reaching to the bottom of the flask, and an outlet tube connected with a suction pump via three boiling tubes each containing 10 ml. of 1 per cent. $KMnO_4$. The flask was immersed in a water bath at 48 to 50° C. and 10 ml. of 10N H_2SO_4 run in from the separating funnel. Air was gently aspirated through the tubes at about six bubbles per second for one and a half to two hours. The tubes were disconnected and 2 ml. 10N H_2SO_4 added to each; the contents were warmed to 70° C. and saturated oxalic acid solution was run in till they were almost colourless. They were then cooled and a blue colour developed with 2 ml. of 7·5 per cent. sodium molybdate and 2 ml. freshly prepared 0·2 per cent. stannous chloride in N/20 HCl. Each tube was made up to 20 ml. with distilled water and the contents of the three were mixed, allowed to stand for ten minutes, and compared in a colorimeter with a standard phosphate solution similarly treated. By this means quantities of volatile phosphorus of the order of 0·005 mg. could be estimated with an accuracy of some 5 per cent. either way.

Zinc was also estimated colorimetrically by the method of Schwaibold *et al.* (1938). In this the material is ashed and extracted with N/2 HCl. The acid extract is shaken with 0·01 per cent. dithizone in carbon tetrachloride to remove copper and the carbon tetrachloride layer separated and discarded. After the addition of 3 c.c. of 20 per cent. sodium potassium tartrate to prevent interference by lead, and neutralisation to thymol blue with 6 per cent. ammonia, the zinc is extracted by dithizone solution, and this, after shaking with 0·08 per cent. sodium sulphide, is compared in a daylight colorimeter with a standard zinc solution similarly treated. The method allows of fairly rapid determination of quantities of the order of 0·01 mg. zinc with an accuracy of plus or minus 10 per cent. Recovery experiments from crop contents carried out with both methods proved satisfactory.

RESULTS

Minimum Lethal Dose

To get an approximate idea of the dosage required, two hens, each weighing about 2 kilos, were given respectively 100 mg. and 10 mg. zinc phosphide. The larger dose proved fatal to one hen

in about four hours, whilst the other hen remained unaffected. Three weeks later the surviving hen was given 50 mg. and succumbed in about eight hours. Five fowls were then given doses of 5·25, 10, 15, 20 and 25 mg. per kilo respectively at 5 p.m. and all died overnight except that given the smallest dose. Two fowls were next given what was intended to be 7 and 9 mg. per kilo. respectively, but owing to an error in weighing the bird (which was noted subsequently at *post-mortem* examination) the first actually received 15·4 mg. per kilo, from which it died, whilst the other recovered after a few days' illness. Two further birds were given 9·3 and 9·5 mg. per kilo respectively, only the larger dose proving fatal.

The minimum lethal dose having apparently been determined as lying between 9 and 10 mg. per kilo body weight, attention was turned to the possibility of " chronic " poisoning occurring with repeated sublethal doses. The fowl which had survived 9·3 mg. per kilo was given a similar amount next day and again the day after ; it was seen to be ailing after the second dose, and succumbed within six hours of receiving the third. Another hen, given daily 5 mg. per kilo survived seven doses before succumbing suddenly. We then commenced a similar experiment with 7 mg. per kilo, but the bird died after the first dose, whilst another fowl, given a single dose of 8 mg. per kilo, remained unaffected.

In all the birds so far examined, both zinc and phosphide had been detected easily in the alimentary canal. It seemed possible, however, that in field cases of poisoning, the phosphide might rapidly disappear from the alimentary tract and not be detectable by analytical methods. Five birds were accordingly given 10 mg. per kilo with the object of keeping them for varying periods after death before making a chemical analysis. The dose was chosen as giving the smallest amount of phosphide which was likely to be found under field conditions, and yet be sufficient to kill the fowls, but contrary to our expectations only two died. The remaining three were left a week to recover and were then given 15 mg. per kilo. Again only two died but the survivor succumbed some ten days later to a dose of 17 mg. per kilo.

The results of our experiments with 25 fowls are summarised in Table I, from which it would seem that the lethal amount of zinc phosphide administered as a single dose lies between 7 and 17 mg. per kilo, and is frequently about 10 mg. per kilo. Two fowls withstood amounts totalling some 30 mg. per kilo given in repeated sublethal doses.

Symptoms

Few symptoms were to be seen. The fowls given the largest doses were dull and depressed with ruffled plumage an hour or so after the capsule had been given. Later, they sat very dejectedly in the corners of their cages, where they usually died in a few hours with no signs of struggling. Those given smaller lethal doses survived

TABLE I

Fowl No.	*Breed and Sex*	*Dose of Zn_3P_2 (mg. per kilo)*	*History*	*Post-mortem Findings*
1	L.S., hen	50	Died in 4 hours	Slight yellowish tint of liver and kidneys. Distended gall bladder. Enteritis, severe in duodenum, extending down two-thirds of the S.I. Distinct smell of phosphine in crop and gizzard—most evident in latter.
2	R.I.R. ×, hen	25	Died in 8 hours	Gelatinous exudate in thorax. Slightly mottled liver ; early mucoid enteritis.
3	R.I.R., cock	25	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
4	R.I.R., hen	25	Died in 12 hours	Fatty liver and heart. Pale pancreas containing white spots. No odour of phosphine.
5	W.L., hen	20	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
6	R.I.R., hen	17	Died in 12 hours. Kept at 70–80° F. for 5 days	No evident lesions. Pronounced P.M. changes. No odour of phosphine.
7	Br.L. ×, hen	15·4	Died in under 16 hours	Small quantity serofibrinous exudate on liver capsule. Venous congestion, small quantity of fluid in pericardial sac.
8	R.I.R., hen	15·2	Died in 12–15 hours	Generalised TB., internal haemorrhage from ruptured liver.
9	R.I.R., hen	15·1	Ill but recovered in about 3 days	—
10	W.W., cock	15	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
11	Bl.L. ×, hen	15	Died in 12–15 hrs. Kept at 50–60° F. for 5 days	Sero-fibrinous exudate over surface of liver. Fluid in abdominal cavity.
12	W.W., cock	10	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
13	W.L., hen	10	Died in 24–36 hours. Kept for 12 days at 40–50° F.	Early P.M. changes. No detectable odour of phosphine. Small amount of fluid in abdominal cavity. Liver congested.
14	R.I.R., hen	10	Died in under 16 hours	Ascites. No other evident abnormalities apart from some tuberculous lesions. No odour of phosphine.
15	R.I.R., hen	10	Unaffected	—
16	W.W., cock	10	Unaffected	—
17	Br.L., hen	10	Slightly ill but recovered in 2–3 days	—
18	Br.L., hen	9·5	Died in 24–36 hours	Ascites. Sero-fibrinous exudate on surface of liver, small quantity of pericardial fluid.

TABLE I—*continued*

Fowl No.	*Breed and Sex*	*Dose of Zn_3P_2 (mg. per kilo)*	*History*	*Post-mortem Findings*
19	Br.L., hen	9·3	Unaffected — used for repeated doses. Died after 3rd dose	Small quantity of pericardial fluid. Liver congested and enlarged. Contents and lining membrane of gizzard were dark green in colour.
20	W.L., hen	9	Recovered after about 4 days' illness	—
21	R.I.R., hen	8	Slightly ill but recovered	—
22	W.L., cock	7	Died in 18 hours	No evident abnormalities.
23	W.L., cock	5·25	Unaffected	—
24	R.I.R. ×, hen	5	Unaffected	—
25	R.I.R., hen	5	Unaffected. Dose given repeatedly for 7 days before being fatal	Small amount serous fluid around pericardial sac and in abdominal cavity. Duodenal enteritis.

some 12 to 36 hours. Their symptoms were similar but less marked and some diarrhoea, with the passage of greenish faeces, was usually observed. Birds given sublethal doses occasionally appeared quite unaffected though the effective administration of the capsule was checked in nearly every case by feeling its passage down the oesophagus into the crop. Some birds, however, sat or stood dejectedly and ate nothing for two to five days before returning to normal. The faeces in these cases were bright green from bile pigments which were excreted in excessive amounts. Recovery eventually appeared to be complete, some of these birds being kept for several months during which time they were perfectly normal. The two " chronic " cases which were given repeated sublethal doses showed nothing abnormal, apart from the passage of large amounts of bright green faeces, until the administration of the final dose, when they rapidly succumbed with symptoms similar to those discussed above.

Lesions

Macroscopic Findings.—Details of the *post-mortem* examinations of the various fowls are set out in Table I. The most usual finding was a variable degree of venous congestion with a certain amount of serous fluid in the pericardial sac and in the abdominal cavity. In a number of cases there was some sero-fibrinous exudate on the liver capsule. Enteritis in the upper part of the small intestine was noted, especially with the larger doses. The characteristic pungent (carbide-like) odour of phosphine was detected in all cases

except where its absence is specifically noted in the table. It was usually most marked in the crop and was noted only occasionally in the gizzard.

Histopathology. — The microscopical picture in the organs examined varied from mild cloudy swelling to fairly severe fatty degeneration, both constantly associated with venous congestion. Cloudy swelling seemed to be a constant feature of acute poisoning with large doses of zinc phosphide.

The liver cells were swollen, with a fine pale granulation of the cytoplasm, but the nuclei appeared normal. The von Küpffer cells had a more plump appearance than usual. Fatty degeneration was seen in birds given repeated sublethal doses. In paraffin sections the cytoplasm was crowded with minute vacuoles and the nucleus showed signs of definite injury, *i.e.*, it was compact, deeply basophilic, and in many cases disintegrated.

Kidney: The cells mainly affected were those of the convoluted tubules, whilst those of the straight tubules showed little change. The glomerular tufts were swollen, and occasionally free red blood cells were seen lying in an extravascular position.

Heart Muscle: A fine granulation of the cytoplasm with loss of striation was the usual picture.

Small Intestine: Cloudy swelling, desquamation of epithelium and a hyperactivity of the mucosal goblet cells were the only noteworthy features.

Pancreas: In the one case examined (No. 4) white spots were found which were identified as necrosed islets of Langerhans.

ANALYTICAL FINDINGS

In Table II the results of analyses of the contents of the alimentary tract of birds which died following a single dose, are compared with those from four control birds which had received no zinc phosphide. The bulk of the material recovered was found in the crop which contained on an average about 40 per cent. of the administered zinc and about 8 per cent. of the phosphide. Only some 6 per cent. of the administered zinc was recovered from the gizzard contents, whilst the intestines contained even less—about 5 per cent. of the total. (If the apparently abnormally high figures obtained with fowl No. 3 be omitted, these averages drop to about 2·5 per cent. and 3·5 per cent. respectively and are obviously of no significance.) Very small amounts of phosphide (*circa.* 0·5 per cent. of the dose given) were found in the gizzard whilst the intestine seldom contained more than the merest trace. Only traces of zinc were found in the control birds and no detectable amounts of volatile phosphorus were obtained except in the crop of No. 1, where a faint trace of blue developed which was barely readable, but was roughly estimated at 0·0015 mg. The two chronic cases induced by the repeated administration of sublethal doses gave findings roughly similar to those obtained with single lethal doses, except for a much poorer percentage recovery of administered material, especially phosphide. This was, of course, due to the fact that probably only phosphine from the last dose given would be available for recovery.

TABLE II

Fowl No.	*Total Zn_3P_2 given (mg.)*	*Zinc found in Contents of*			*Volatile P found in Contents of*		
		Crop (mg.)	*Gizzard (mg.)*	*Intestine (mg.)*	*Crop (mg.)*	*Gizzard (mg.)*	*Intestine (mg.)*
Single Doses							
3	75	1·86	12·7	6·95	—	—	—
2	50	25·2	1·15	1·92	—	—	—
4	61	13·7	0·33	0·43	2·34	0·049	0·006
5	48	19·3	3·74	—	—	—	—
10	36	9·37	0·84	—	—	—	—
8	28·5	11·3	0·65	1·63	0·21	0·007	Nil
12	25	9·1	0·09	—	—	—	—
14	21	16·8	0·13	0·9	0·052	0·037	Nil
7	20·7	9·7	0·28	0·7	0·53	0·083	0·019
18	17	15·8	0·08	0·52	0·226	0·022	0·009
22	16·4	9·45	0·11	0·92	0·106	0·007	Nil
Repeated Doses							
19	3 × 9·3	7·1	0·27	2·2	0·118	0·023	0·033
25	7 × 5	9·64	0·19	0·4	0·023	0·057	Nil
Controls							
IX	Nil	0·17	0·38	1·1	0·0015	Nil	Nil
XIII	Nil	0·27	0·15	0·69	Nil	Nil	Nil
XVIII	Nil	0·11	Nil	0·37	Nil	Nil	Nil
XX	Nil	0·78	0·15	0·09	Nil	Nil	Nil

TABLE III
AVERAGE ZINC CONTENT OF TISSUES

	Liver (mg. per 100g)	*Gall Bladder and Contents (mg.)*	*Kidneys (mg.)*	*Heart (mg.)*	*Lungs (mg.)*	*Breast Muscle (mg. per 100 g.)*	*Intestinal Wall (mg.)*
Fowls given single lethal doses (12) ...	3·04	—	—	—	—	—	0·91
Fowls given repeated doses (2) ...	3·6	0·05	0·3	0·27	0·16	0·38	0·6
Controls (6)	5·98	0·05	0·5	0·13	0·42	0·57	0·75

TABLE IV
EFFECT OF P.M. CHANGES

Fowl No.	*Dose of Zn_3P_2 given (mg.)*	*Time between Death and Autopsy (days)*	*Temp. during Storage °F.*	*Zinc found in Contents of*			*Volatile P found in Contents of*		
				Crop (mg.)	*Gizzard (mg.)*	*Intestine (mg.)*	*Crop (mg.)*	*Gizzard (mg.)*	*Intestine (mg.)*
13	19	12	40–50	7·55	0·11	0·27	0·022	0·01	Nil
11	31·5	5	50–60	2·68	0·44	2·0	0·026	0·034	0·015
6	35·7	5	70–80	24·2	2·24	0·39	0·083	0·02	Nil

We thought originally that some of the zinc might be absorbed and stored in tissues such as the liver, and that if the results were significant, tissue analysis might provide a rapid and reliable means of diagnosis. The results of analysis of the tissues summarised in Table III showed, however, that there was no storage of zinc in either acute or chronic cases—in fact, the livers of birds which had received no zinc phosphide contained significantly larger amounts of zinc than we found in the livers of our experimental fowls. (This might be due to the excessive secretion of bile occurring in dosed birds removing some of the small amounts of zinc normally present.) The apparent differences in zinc content of kidneys and lungs between control and treated birds were not statistically significant. Analysis of faeces passed during the experiments indicated that there is a rapid excretion of the administered zinc, and in cases where repeated sublethal doses were given, faeces collected daily and analysed showed a steady excretion of the bulk of the administered zinc, about 60 per cent. of which was so recovered.

The effect of *post-mortem* changes on the recovery of zinc and volatile phosphorus was determined with three birds which were kept for varying periods after death before being autopsied (Table IV). The recoveries of zinc from crop, gizzard and intestines were of the same order as for birds examined soon after death, being on the average 50 per cent., 3·5 per cent., and 4 per cent. respectively of the doses administered. But as was expected, the recoveries of phosphide were considerably less, that found in the crop being only some 0·6 per cent. of the dose given, whilst the gizzards had only half as much and the intestines little or none. Nevertheless, in every case, in spite of *post-mortem* changes, it was still possible to detect significant amounts in the crop contents though the smell of phosphine was masked by other odours.

DISCUSSION

Our observations confirm field experience that zinc phosphide is a substance highly lethal for poultry, a tablespoonful of the rat bait normally used by pest officers containing sufficient material to poison at least 50 to 60 birds. There was nothing very characteristic about the symptoms which were probably similar to those found in any form of acute poisoning, though the somnolent condition which in some cases preceded death may have been analogous to the narcosis reported by Ingram (1945) in a colt which died from a similar cause. Though symptoms of asphyxia are common in hydrogen phosphide poisoning in man, we found no suggestion of them in our experimental birds. *Post-mortem* changes were also not very characteristic as they were suggestive simply of acute poisoning. We did not see any signs of the liver necrosis recorded by Hare and Orr (1945). At *post-mortem* examination the odour of phosphine is probably the best general guide though it may be missed if the dose is small or if the autopsy is long delayed. There should be no difficulty,

however, in finding phosphine in the crop by analytical methods in the majority of cases even after some delay, provided an unopened crop is received and a sensitive method is used. For diagnostic purposes this need not be quantitative—rapid distillation of the phosphine from boiling acid solution into permanganate and the development of a blue colour by any of the usual methods for the colorimetric determination of phosphorus should suffice. Hydroquinone, amino-naphthol-sulphonic acid or stannous chloride may all be used though stannous chloride is to be preferred as it is much the most sensitive. Even after considerable *post-mortem* change has taken place, a detectable blue can be obtained by such methods. One obstacle to such determinations lies in the fact that free phosphorus gives the same reaction and, while the *post-mortem* changes in well-marked cases of phosphorus poisoning are somewhat different from those found with zinc phosphide, it seems just possible that quantities of free phosphorus too small to give detectable Mitscherlich or Scherer reactions, might be confused with zinc phosphide. There ought to be no difficulty in detecting zinc in the crop contents, as in all probability there would seldom be less than 8 or 9 mg. present and often considerably more. We have used with considerable success a qualitative ammonium mercuric thiocyanate test, suggested by Watkins (personal communication), but it involved a rather tedious separation from other elements and we find the dithizone method described above both simple and effective. For legal purposes, presumably, evidence of both zinc and phosphine would be desirable before stating categorically that zinc phosphide was present, and in such a case a quantitative estimation of zinc would often give a very good idea as to whether sufficient had been consumed to cause death. The routine we have adopted in suspected cases, therefore, is to carry out both a qualitative test for phosphine and a quantitative determination of zinc on the crop contents.

SUMMARY

A method is described for the quantitative estimation of very small quantities of phosphide.

The lethal dose of zinc phosphide for fowls has been determined as lying between 7 and 17 mg. per kilo body weight, being frequently about 10 mg. per kilo.

Chronic effects have been produced by repeated sublethal doses.

Symptoms, lesions and analytical findings in experimental zinc phosphide poisoning are described.

The significance of these findings in toxicological work is discussed.

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REFERENCES

Elmore, J. W., and Roth, F. J. (1943). *J. Ass. off. agr. Chem., Wash.*, **26**, 559.
Hare, T., and Orr, A. B. (1945). *Vet. Rec.*, **57**, 17.
Ingram, P. L. (1945). *Ibid.*, **57**, 103.
Schwaibold, J., Bleyer, B., and Nagel, G. (1938). *Biochem. Z.*, **297**, 324.

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SOME OBSERVATIONS ON THE POISONOUS PROPERTIES OF BRACKEN (*PTERIS AQUILINA*)*

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INTRODUCTION

Cases of fatal illness in cattle due, it was believed, to eating bracken, were first recorded by Penberthy (1893) and Storrar (1893), both of whom had investigated outbreaks of an apparently new disease which occurred during the long drought experienced that year. There seems little doubt that these authors' suspicions regarding the origin of the condition were well founded for the symptoms and lesions which they described correspond closely to those which can be set up experimentally by feeding bracken to cattle. Proof of this was soon forthcoming for in an editorial article published in this journal in 1894 which revealed on the authority of the late Nicholson Almond, F.R.C.V.S., that the disease had been reproduced by feeding animals on bracken chopped up with straw and gradually withdrawing the straw until the diet was composed solely of bracken and turnips. This was soon followed by the death of three of the animals and the serious illness of two others. The results of this experiment and those obtained subsequently by other workers are a sufficient indication that bracken is to be regarded as a potential danger to cattle, but nevertheless the toxicity of the plant has been the subject of much discussion. The feeding trials carried out have been on a rather limited scale and in some instances have yielded negative results. Moreover, as regards the high temperature which is always present, the latent period observed in many cases and the nature of the lesions produced, bracken poisoning resembles various bacterial diseases, for example, those caused by organisms of the Clostridium group. Considerations of this kind gave rise to the suggestion that the disorder was possibly due to a bacterial agent, but Stockman (1909, 1916, 1917, 1922), Bosshart and Hagan (1920), Hagan (1922, 1925) and Kerdiles (1927, 1929), in spite of careful investigations, could find no evidence to support this view; it is now accepted that organisms found in the tissues of animals dead of bracken poisoning are agonal or *post-mortem* invaders.

This paper records the results of an experiment in which two heifers were used for a feeding trial with bracken, and includes some observations on the poisonous properties and chemical composition of the plant.

FEEDING EXPERIMENTS

As preliminary trials had shown that guinea-pigs were not suitable experimental animals in which to study the toxic effects of bracken, and sheep could not be induced to take it, a feeding experi-

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ment was carried out on two heifers, to ascertain whether or not the result would confirm the findings of others as to the toxicity of the plant and whether the poison was capable of changing the blood picture in a way which might serve as an aid to diagnosis.

Freshly cut bracken gathered at weekly intervals from a colony near Brandon in Suffolk was fed to two 18-months-old heifers, one of which was given bracken only and the other a diet consisting of equal parts of bracken and normal food, the latter being made up of hay and straw chaff, $3\frac{1}{2}$ parts; decorticated ground nut cake, $1\frac{1}{4}$ parts; bran, 1 part; sugar beet pulp, 1 part; hay, 7 parts. They were housed in loose boxes and fed on normal food for four days before the experiment was commenced on July 15th. Heifer No. 1 received 20 lb. chaffed bracken per day as its sole diet, while No. 2 was allowed 7 lb. of the above mixture and 7 lb. of chaffed bracken. Peat moss was used instead of straw for bedding as it was not likely to be eaten. The animals soon became accustomed to the experimental diet and ate it fairly readily. Any residue from the previous day's feeding was collected and weighed each morning, so that the actual amount of food consumed by each could be recorded. The temperature, pulse and respirations were taken twice daily, at 10.30 a.m. and 3.30 p.m., and samples of blood were collected twice a week from the jugular vein, in order that the following determinations might be made: red cell count, percentage haemoglobin, sugar, alkali reserve, non-protein nitrogen, Ca, inorganic P and Mg.

Heifer No. 1.—Eight days after the experiment had commenced this animal was observed to be constipated and dull. On the 13th day an excess of mucus was noticed for the first time in the faeces, which were normal in colour but hard in consistency, and soon assumed a dark, almost black, appearance. The animal gradually

TABLE I
RECORD OF TEMPERATURE, PULSE AND RESPIRATIONS OF HEIFER NO. 1 FROM SEPTEMBER 22ND TO 28TH

Date	*Temperature ° F.*	*Pulse per Minute*	*Respirations per Minute*	*Remarks*
22/9	103·2	20	15	Apparently normal but dull.
23/9	105·2	24	15	Bleeding from nose.
24/9	10.30 a.m. 104·8	26	13	Bleeding from nose less profuse.
	3.30 p.m. 106·0	19	14	
25/9	10.30 a.m. 107·2	42	13	Disinclined to rise. Pulse irregular, bleeding from nose.
	3.30 p.m. 107·6	58	15	Faeces give strong positive benzidine test for blood. Urine normal.
26/9	106·9	50	12	Bleeding from anus.
27/9	105·3	48	14	Offensive smelling, bloody diarrhoea.
28/9	104·7	46	10	Moribund, stertorous breathing. Killed.

lost condition but its pulse, respiration and temperature remained normal during the next eight weeks, and no marked symptoms were observed until September 22nd, when the temperature rose to 103·2° F. The following day a trickle of bright red blood from the nose was seen and the temperature reached 105·2° F. From then onwards the animal became steadily worse. The course and symptoms shown during the last few days of the illness are recorded in Table I.

On the evening of September 28th, as the animal was evidently in great pain, it was destroyed and examined *post mortem* next morning by Dr. Innes, who has kindly supplied the following particulars of his findings :—

" Widespread multiple, diffuse, subcutaneous and intramuscular haemorrhages throughout the body, frequently spreading along the fascial planes ; occasional foci of gelatinous oedema in association with the haemorrhages ; widespread, diffuse, subserous haemorrhages (peritoneum, pleura, pericardium, subserosa of the gut and uterus) ; gross diffuse haemorrhages in the wall of the bladder and omasum ; multiple petechiae in the intestinal and abomasal mucosa ; petechiae and haemorrhagic infarcts in both kidneys ; " acute splenic tumour," with subcapsular and pulp haemorrhages ; fatty liver with advanced *post-mortem* emphysema ; hypostasis and oedema of the left lung ; two large diffuse foci of serofibrinous pleurisy with adhesions on the right side ; myocardial haemorrhages."

Such a picture is consistent with the effects of toxic substances circulating in the blood stream.

Bacteriological Examination

Cultures were made from various organs of the body but the only bacteria isolated were *post-mortem* invaders commonly found in bovine carcases which have lain for some hours after death. Some delay was unavoidable in this case, as the animal had to be destroyed in the evening and could not be examined until the following morning. There was, however, no indication that bacterial infection played any part in causing the animal's death.

The heifer ate 915 lb. of bracken in 74 days (average 12·4 lb. per day) but showed no definite signs of illness until it had been on experiment for 68 days. The symptoms were typical of those described by Stockman (1909), Hagan and Zeissig (1927) and Kerdiles (1927), but they took much longer to develop than in Stockman's experiments. Stockman, however, worked with younger animals and may conceivably have used bracken of higher toxicity. The lesions also were in agreement with those found in bracken poisoning, and it was concluded, therefore, that death had resulted from the consumption of the fern.

Heifer No. 2.—The heifer fed on a ration containing 50 per cent. of bracken remained normal throughout the experiment ; it lost only 59 lb. in weight, whereas heifer No. 1 lost 191 lb. Towards the end of the experimental period its faeces contained an excess of mucus, but at no time was it badly constipated. It was kept on the diet until October 2nd, when, as the bracken was showing signs

of withering, the animal was turned out to pasture, and kept under observation for a further period during which it appeared to be normal and gradually gained in weight. It was killed on October 26th and its organs were found to be healthy on *post-mortem* examination. This animal ate 678 lb. of bracken in 78 days, average 8·7 lb. per day, without showing any symptoms of poisoning.

Examination of the Blood.—Repeated determinations made during the experimental period failed to reveal any significant change in the composition of the blood produced by the feeding of bracken. Even the onset of symptoms in heifer No. 1 was not accompanied by an alteration in the blood picture, which remained normal almost up to the time that the animal became moribund.

The sample of bracken used in this experiment killed the heifer which was fed on it as its sole diet for a prolonged period. When consumed for a similar length of time in smaller, though still considerable quantities (8·7 lb. per day) mixed in equal proportions with normal food, it caused no apparent harm to an animal of the same age. It is uncertain how this difference should be explained for the relative importance of various factors which may play a part in experimental bracken poisoning in cattle could only be determined by feeding trials on an adequate scale, under carefully controlled conditions. The existing data which are summarised in Table II, concern a small number of animals only and as the procedure adopted by different investigators has been by no means uniform, it is difficult to draw hard and fast conclusions by comparing their results.

TABLE II
SUMMARY OF FEEDING EXPERIMENTS WITH BRACKEN CARRIED OUT BY VARIOUS WORKERS

Investigator	*Animal*	*Age*	*Amount Bracken fed (lb.)*	*Length of Experiment in days*	*Amount fed per day (lb.)*	*Result*
Stockman (1917)	Bull calf	8 months	260	26	10	Died.
Stockman (1922)	Calf	4 months	160	30	5·3	Died.
Stockman (1922)	Calf	3½ months	112	30	3·7	Died.
Hagan (1927)	Cow	6 years	—	52	—	Lived.
Hagan (1927)	Bull	1½ years	—	52	—	Lived.
Hagan (1927)	Bull calf	8 months	—	66	—	Died.
Bruce (1927)	Heifer	3 years	578	262	2·2	Lived.
Bruce (1927)	Calf	3½ months	264	30	8·8	Lived.
Bruce (1927)	Heifer	15½ months	495	40	12·4	Lived.
Shearer	Heifer	18 months	915	74	12·4	Died.
Shearer	Heifer	18 months	678	78	8·7	Lived.

It will be seen, however, that whereas Stockman was able to kill calves of three and a half to eight months old quite readily with bracken, Bruce (1927) failed to do so although he fed a larger amount

of the plant. This appears to indicate a difference in the toxicity of the samples used in the two cases. Again, the results in general provide some indication that the age of the animal may be of importance but much more evidence is needed concerning this and various other factors which have to be taken into consideration, such as the total amount of the plant consumed and the daily intake.

Chemical Composition

A search of the literature showed that no systematic chemical examination of bracken had been undertaken. Some analyses had been carried out to determine the nutritive value of the plant, and Greshoff (1908) had mentioned the presence of a cyanogenetic glycoside in the young shoots. Many textbooks relate the poisonous properties of the fern to the presence of Pteritannic acid, a substance described by Watts (1866), who, however, detailed its preparation from *Aspidium Filix Mas*, not from *Pteris Aquilina*.

In view of this lack of knowledge of the chemical composition of the plant, a search was made for a substance to which its poisonous properties could be ascribed. Repeated attempts to reveal the presence of alkaloids, phytotoxins, saponins or glycosides by the usual methods of examination were unsuccessful and attention was then directed to the type of tannin contained in the plant.

Examination of the Tannin present in Bracken

The tannin from a strong aqueous solution of bracken was precipitated with a suspension of copper carbonate. The precipitate was filtered off, washed three times with water and an aqueous suspension prepared. After filtration the residue was taken up with alcohol and precipitated by the cautious addition of 10 per cent. NaOH. The precipitate was filtered off and the aqueous solution gave the following reactions :—

Addition of a few drops of $FeCl_3$ solution gave an intense green coloration.

A light yellow precipitate was produced by the addition of bromine water.

The solution precipitated quinine from a solution of its hydrochloride.

Gelatine was precipitated from its aqueous solution.

On boiling an aqueous solution of the tannin with dilute mineral acid (HCl or H_2SO_4) a red precipitate was formed—a phlobaphene.

The formation of a green colour with $FeCl_3$ and the production of a phlobaphene on boiling with mineral acids shows that it is a catechol tannin. The amount of tannin, like that of other constituents of bracken, varies at different stages of growth. In order to ascertain the extent of the variation which occurs, samples gathered from the Newmarket colony at monthly intervals throughout the growing season were analysed, using the methods employed by the Rowett Institute, Aberdeen. The results which are given in Table III show that the plant is evidently of low nutritive value on account of

the small quantity of crude protein and the excess of fibre and tannin which it contains.

TABLE III

VARIATION IN THE CHEMICAL COMPOSITION OF BRACKEN THROUGHOUT THE GROWING SEASON. PERCENTAGES EXPRESSED ON DRY WEIGHT BASIS

Mth.	*Crude Protein*	*Total Ash*	*Insol. Ash*	*Sol. Ash*	*CaO*	*Fat*	*Fibre*	K_2O	Na_2O	P_2O_5	*Tannin**	*Remarks*
May	21·20	8·14	0·53	7·61	0·28	2·27	15·62	2·93	0·98	1·20	1·63	Young shoots, fronds not developed.
June	13·50	5·23	0·64	4·59	0·43	1·38	26·44	1·81	0·28	0·71	2·81	Fronds just appeared.
July	9·37	6·45	0·75	5·70	0·67	1·18	31·43	1·96	0·34	0·56	7·00	Plant at maximum growth.
Aug.	—	5·78	0·79	4·99	0·80	2·53	29·95	1·82	0·47	0·37	6·04	Tips of fronds beginning to turn brown.
Sept.	6·68	5·63	1·38	4·25	0·86	2·15	33·36	1·46	0·30	0·23	6·26	Plant commencing to wither.
Oct.	5·0	5·07	1·52	3·55	0·78	2·06	39·77	0·82	0·31	0·19	0·44	Plant quite brown and hard.

* The tannin was estimated by the method described by Rosenthaler (1930)

DISCUSSION

It would appear that bracken gives rise to fatal results only when consumed in large quantities over a long period. The presence of tannin in the fern is conducive to constipation, a symptom which was observed in the case reported in this paper, in which it set in soon after the commencement of the experiment and persisted almost to the time of the animal's death. Other tannin-containing plants have been found to cause illness in cattle, the general action being that of an astringent. Among these are acorns, oak, beech and bracken, but, apart from constipation, the symptoms and lesions which they produce have little, if anything, in common. It has been suggested by Lander (1912) " that specially active forms of tannin or unrecognisable active principles may be responsible, but positive evidence is lacking." Such a source of irritation may allow an active principle to be more readily absorbed and the length of time necessary for symptoms to develop may be correlated with the time required to produce the requisite increase in the permeability of the gut wall.

The poison contained in bracken has been described as cumulative because of the large amounts of fern which have to be consumed before symptoms develop. Cumulative poisons, however, usually produce characteristic lesions of a chronic nature, whereas those of bracken poisoning are essentially of an acute type.

Natural cases of bracken poisoning generally occur when the supply of herbage is scanty owing to the prolonged period of drought. Animals which are forced to exist mainly on a diet of bracken must suffer from some degree of malnutrition, owing to the low nutritive value of the plant. A low plane of nutrition would tend to lower the resistance of the animal to the poisonous principle which, in bracken, may be a decomposition product of the catechol tannin.

SUMMARY

A fatal result was produced in a heifer by a diet composed solely of bracken. The symptoms and *post-mortem* lesions described by former workers were observed. No change in the cellular or chemical composition of the blood was produced which would serve as an aid to diagnosis of bracken poisoning.

Another heifer, whose diet was composed of equal parts of bracken and normal food, ate three-quarters of the amount of bracken which proved fatal to the other animal over a similar period of time, but showed no harmful effect.

Chemical analysis of the plant during the growing season showed it to be of low nutritive value and to contain varying amounts of a catechol tannin, but no other substance to which its poisonous properties could be ascribed, was found.

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REFERENCES

———(1894). *J. comp. Path.*, **7,** 165.
Bruce, E. A. (1927). *Bull. 88. Canadian Dept. Agric.*, pp. 7-12.
Bosshart, J. K., and Hagan, W. A. (1920). *Cornell Vet.*, **10,** 102.
Greshoff, M. (1908). *Pharm. Weekbl.*, **45,** 770.
Hagan, W. A. (1922). *Cornell Vet.*, **12,** 43 ; (1925). *Ibid.*, **15,** 326.
Hagan, W. A., and Zeissig, A. (1927). *Ibid.*, **17,** 194.
Kerdiles, J. Y. (1927). *Thèse Vétérinaire,* Paris ; (1929). *Rev. gen. Med. vet.*, **38,** 1.
Lander, G. D. (1912). *Veterinary Toxicology*, p. 271. Baillière, Tindall & Cox : London.
Penberthy, J. (1893). *J. comp. Path.*, **6,** 266.
Rosenthaler, L. (1930). *The Chemical Investigation of Plants*, p. 118. G. Bell & Sons, Ltd. : London.
Stockman, S. (1909). *Report of the Chief Veterinary Officer to the Ministry of Agriculture* ; (1916). *J. comp. Path.*, **29,** 95 ; (1917). *Ibid.*, **30,** 311 ; (1922). *Ibid.*, **35,** 273.
Storrar, D. M. (1893). *Ibid.*, **6,** 276.
Watts, —. (1866). *A Dictionary of Applied Chemistry*, Vol. 4, p. 745.

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NEOPLASTIC DISEASE OF THE FOWL WITH SPECIAL REFERENCE TO ITS HISTORY, INCIDENCE AND SEASONAL VARIATION*

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INTRODUCTION

That the incidence of tumorous conditions is high in fowls compared with other animals is well known to comparative pathologists, who, recognising the peculiar liability of avian tissues to undergo neoplastic changes, make use of this in the experimental induction of tumours by chemical means. The transmissibility of some spontaneous tumours and leukaemias, either by tissue inoculation or cell-free filtrate, is also well established since Peyton Rous's (1910) discovery, and a large amount of experimental work has subsequently been accomplished in this field, yielding information of first-class importance and giving an impetus to cancer research, in which branch particular mention must be made of Fould's work (1934, 1940). In contrast, little has been published on the incidence of spontaneous tumours in fowls generally. Only two articles (Goss, 1940, and Bullis and Olson, 1943) give figures for the comparative incidence in the more common breeds, and there is a scarcity of information recorded on the incidence in fowls generally of the various specific neoplastic conditions and on organ distribution and seasonal incidence.

To estimate the incidence of tumours in fowls generally is obviously very difficult for it entails the maintenance of large flocks of healthy birds under observation, all of which would have to be subjected to a very thorough examination when they died or were destroyed. For this reason, the somewhat meagre information on the subject relates almost entirely to the proportion in which it is observed in disease in general in the *post-mortem* examination records of poultry laboratories engaged in routine diagnosis. The value of the few attempts to discover the absolute incidence of the tumours has usually been limited by failure to submit the material to histological examination. Some attempts at classification have been made but these are not of much significance because they have been based on macroscopic observations, faulty histological diagnosis or, very frequently, obsolete classification. It is certain that many undifferentiated carcinomata primary in the ovary have been classed as "fowl paralysis tumours," lymphocytoma and so on; that many cases of erythroleucosis have been called lymphatic leukaemia, and that granulomata have been variously diagnosed as endotheliomata,

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peritheliomata or round-cell sarcomata, depending on the observed abundance of proliferating capillaries or the degree of infiltration with round cells. Critical examination of the gross and histological characters, special staining techniques and a study of the distribution of the tumour should suffice in most cases to establish its identity. Routine bacteriological examination of all suspicious growths, together with the employment of bacterial stains, would eliminate most of the granulomata.

Revision of nomenclature has invalidated much of the early work on the relative incidence of specific tumours. The term " sarcoma " is now entirely confined to use as a suffix to a definite word, *e.g.*, fibro-sarcoma, osteo-sarcoma, and so on ; and there is now a strong tendency to discard the term completely and to speak of a fibroblastoma, osteoblastoma, etc. In the light of present knowledge on the histogenesis of tumours the term " round-cell sarcoma," so commonly used in the past, is obviously no longer desirable. Formerly, however, " round cell sarcoma " was frequently the diagnosis for tumours which are now classified as lymphoblastoma and were therefore included among the connective tissue tumours.

One cannot be certain, moreover, from examination of the figures for the incidence of leucotic tumours, whether those growths which are manifestations of the visceral form of fowl paralysis have been included or not. Histologically such growths, even when they occur in birds, it should be emphasised, with evident neurolymphomatosis, are indistinguishable from multiple discrete lymphocytomata and have a similar distribution, although, as will be mentioned later, it is possible that the organ incidence differs. Therefore, it seems reasonable to include such tumours among the lymphoblastomata, although in subsequent tables they have been placed in a separate column for the sake of comparison and clarity.

Finally, neglect to examine the brain as a routine at autopsy no doubt accounts for the rarity of nerve tissue tumours reported in the literature. Without a careful examination of the brain, nervous symptoms cannot be dismissed as attributable to fowl paralysis or dietetic deficiency.

The earliest descriptions of neoplastic disease in the domesticated fowl appear to have been by Roloff (1868) and Caparini (1896). There was then a lapse of several years, during which no publications are traceable, until an article by Butterfield (1905). This was followed by three articles in 1907 by Kon, Ehrenreich and Warthin respectively, and by Ellermann and Bang's articles in 1908–09. Other early contributors were Tyzzer and Ordway (1909), Hirschfeld and Jacoby (1909 and 1912), and Schmeisser (1916). Of these writers, Ehrenreich (1907) attempted to ascertain the incidence of neoplastic conditions in the fowl, examining 4,000 carcases destined for human consumption and finding seven malignant tumours. All the other papers dealt exclusively with leucotic conditions. Bürger (1914) was the only other of the earlier workers to attempt to find the incidence of tumours in fowls, recording among the 852 birds examined that 12 (1·4 per cent.) had tumours and that seven of these affected the ovary. To Curtis (1915), however, must belong the credit of having been the first

to make an accurate assessment of the frequency of tumours in the domestic fowl. A flock of 880 birds was kept under observation and all that died were examined *post-mortem*. The incidence of neoplastic disease in the whole flock was 8·98 per cent. and a distinct rise was noticed among the older birds. Figures are given for organ distribution but unfortunately the tumours were only classified as solid or cystic, without histological examination.

Other workers who have investigated the incidence of neoplastic disease in fowls or who give figures from which the incidence can be computed, are :—

Worker	*Number of Fowls Examined*	*Percentage affected*
Klee (1917)	611	14·5
Lentz (1922)	297	2·6
N.J. State—		
(1924)	142	11·9
(1925)	106	6·6
	861 (pullets)	7·5
Matheson (1927)—		
Flock A	424	3·3
Flock B	402	13·3
Flock C	516	7·1
Kerr (1928)	460	9·8
N.Y. State Vet. Coll. (1929–30) ...	1,436 (60% adults)	14·6
	1,350 (40% adults)	4·2
Malke (1930)	858	3·8
Babic (1931)	647	6·4
Bullis and Olsen (1943)	2,304	12·9

Matheson and Wilson (1933) give an incidence of 12·1 per cent., the estimation being based on total death figures over a period of 19 years. Goss (1940) had six flocks under observation. In flock A, the incidence of tumours during a period of three years was 16·48, 14·16 and 16·69 per cent. respectively. Flocks B, C, D and E were under observation for one year only and showed 10·62, 20·39, 23·18 and 24·61 per cent. respectively. Flock F showed 24·7 per cent. in White Leghorns, 23 per cent. in Rhode Island Reds and 52·81 per cent. in Barred Plymouth Rocks. The totals show that neoplastic disease occurred in 1,446 fowls (19·51 per cent.) out of 7,408 examined. Dudley, Dobson and Gordon (1941), analysing their figures for the years 1929 to 1937, show that 8 to 10 per cent. of the total mortality was due to tumours of the reproductive tract. The incidence of tumours in other organs varied from 3 to 12·7 per cent. while the leucotic complex accounted for 4·6 to 8·6 per cent. of the total mortality.

It will thus be seen that the incidence of neoplastic disease as reported by various workers during the last four decades varies between 0·17 per cent. (Ehrenreich, 1907) and 19·5 per cent. (Goss, 1940).

In view of this wide variation, it was thought that an examination of the figures of the Poultry Diseases Department of the Royal (Dick) Veterinary College might yield interesting results, especially as particular attention has been paid to neoplastic diseases in fowls during the past five years. During this time, every bird having tumours or leucosis was submitted to a detailed autopsy and all the

main organs, whether grossly abnormal or not, were submitted to histo-pathological examination.

MATERIALS AND METHODS

The material used in this investigation was obtained from fowls sent to the laboratory for autopsy during the five-year period 1939–1944. The birds were generally dead when received but a number of living fowls were examined in which neoplastic disease was diagnosed and in these cases it was possible to submit material to cytological examination, a procedure which is not possible with the ordinary *post-mortem* material of one or two days' duration. At the same time the latter material was generally sufficiently fresh for all routine histo-pathological study, and the few fowls with tumours which were in such a state of decomposition when received as to make histological examination worthless were less than 1 per cent.

Complete records were kept, including breed, sex, age (when known), whether destroyed or died, organ distribution and appearance of tumours, histological details and photographs. Tissues were fixed in 10 per cent. formol-saline and embedded in paraffin wax. Sections were usually cut at 5 to 8 μ and stained with Harris' alum haematoxylin and eosin, unless they were to be photographed, when Erlich's acid haematoxylin was substituted, as it was found to be a more intense stain and therefore more suitable for photography. If considered necessary, special fixatives and special stains were used. In a large number of cases, the distribution of the reticulum was studied, using Laidlaw's silver-carbonate technique. In all leucotic conditions, blood films and marrow or organ impressions were examined after staining with May-Grünwald-Giemsa. Marrow and organ impressions stained better if they were dipped in absolute alcohol for a few minutes, then dried and stained. This was attributed to the dissolving out of intracellular lipoids by the alcohol, thus allowing better penetration by the stain. In cases of suspected granulomata, bacteriological methods frequently gave useful information. Bacterial stains were also used on sections from such cases. In this way, many lesions liable to be confused with true neoplasms were eliminated.

CLASSIFICATION

No entirely satisfactory scheme for the classification of neoplasms is yet available, and none will be available until the exact histogenesis of all tumours is known. In avian pathology difficulties of classification are increased by the occurrence, as already mentioned, of lymphoid tumours in association with a specific disease, *viz.*, fowl paralysis, and thus the evidence for and against the inclusion of fowl paralysis tumours with other lymphoid neoplasms was carefully examined. It was decided to include them and designate the group "lymphoblastomata." Similar difficulties exist with erythroleucosis. Some evidence, based chiefly on Furths' (1931) observations that the virus isolated from erythroleucotic fowls sometimes produced erythroleucosis and sometimes myelogenous leucosis, suggests that this condition should be included in the myeloblastomata. Certainly a mixed myeloid and erythroleucosis is not infrequently seen in the same bird. On the other hand, Jordan

(1936) claims to have shown that avian red blood cells develop from small lymphocytes, which he therefore prefers to call lymphoid haemoblasts. If this is true, erythroleucosis should be grouped with the lymphoblastomata, but with such conflicting evidence it is safest at present to place erythroleucosis in a provisional group by itself termed erythroblastomata.

That all three leucotic conditions in the fowl are related is suggested by the fact that in almost all reptiles, birds and mammals, the erythrocytopoietic and granulocytopoietic tissues are intimately associated. In many species of sub-reptilian vertebrates, these tissues are located in different organs : the Salamander, for example, has erythrocytopoietic tissue in the spleen and granulocytopoietic tissue in the liver. But in each of these organs, the haemopoietic tissue is associated with identical lymphoid tissue, so that lymphoid cells may be ancestral to both erythrocytes and granulocytes (Jordan, 1936).

The classification adopted (Table I) is essentially that of Mallory (1914), modified to conform with avian neoplasia. Mallory's classification is based on the fact that tumours can originate from any of the sixteen or so different types of cell constituting the healthy animal body with a separate histological identity, which normally arise during embryogenesis.

RESULTS AND DISCUSSION

The following is a list of the various types of neoplastic disease found during the investigation. (It will be noted that owing to uncertainty as to their classification, thymoma, adrenal-cortical tumour, osteoclastoma and thecoma have been omitted from the classification, Table I.)

Adenocarcinoma fibromatosum.	Erythroleucosis	Fibroma
Adenocarcinoma leiomyomatosum	Myeloid leucosis	Fibro-sarcoma.
Hepatocellular carcinoma.	Lymphoid leucosis	Histiocytic sarcoma
Cholangiocellular carcinoma	Myelocytoma	Reticulum-cell sarcoma
Hepatoma (adenoma)	Lymphocytoma	Fibro-leiomyoma
Benign cystadenoma of lung	Thymoma	Thecoma (theca cell tumour)
Anaplastic carcinoma	Adrenal-cortical tumour of ovary	Astrocytoma
Haemangio - endothelioma	Embryonal nephroma	Spongioblastoma
Lymphangio - endothelioma		Neuroma
		Osteoclastoma
		Osteogenic sarcoma
		Chondroma

TABLE I

CLASSIFICATION OF AVIAN NEOPLASTIC CONDITIONS
(MODIFIED AFTER MALLORY)

Type Cell	*Normal Product*	*Tumour*
Fibroblast	Connective tissue	Fibroblastoma, including fibroma, fibro-sarcoma, reticulo-sarcoma and mixed-cell tumours (histiocytic and fibroplastic sarcoma).
Leiomyoblast	Smooth muscle	Leiomyoblastoma, including leiomyoma (fibro-leiomyoma of broad ligament) and leiomyosarcoma.
Endothelioblast	Blood and lymph vessel endothelium	Endothelioblastoma, including haemangio-endothelioma and lymphangio-endothelioma.
Lymphoblast	Lymphocyte	Lymphoblastoma, including lymphocytoma or aleukaemic lymphoid leucosis, lymphatic leukaemia and fowl paralysis tumour.
Erythroblast	Red blood cell	Erythroblastoma, aleukaemic or leukaemic (erythroleucosis).
Myeloblast	Myelocyte	Myeloblastoma, including myelocytoma and myeloid leucosis, leukaemic and aleukaemic.
Glioblast	Neuroglia cells	Glioblastoma, including glioma and gliosarcoma.
Osteoblast	Bone tissue	Osteoblastoma, including osteoma and osteogenic sarcoma.
Chondroblast	Cartilage tissue	Chondroblastoma, including chondroma and chondro-sarcoma.
Epithelioblast	Epithelium	Epithelioblastoma, including papilloma, adenoma and carcinoma.
Neuroblast	Nerve cell	Neuroblastoma, including neuroma.
Foetal anlage	Normal organ, *e.g.*, kidney	Misplaced and disordered growth producing teratoma, *e.g.*, embryonal nephroma.
	Not Observed	
Mesothelioblast	Mesothelium of serous surfaces	Mesothelioblastoma, including mesothelioma.
Myxoblast	Mucous connective tissue	Myxoblastoma, including myxoma and myxosarcoma.
Lipoblast	Fat tissue	Lipoblastoma, including lipoma and liposarcoma.
Melanoblast	Pigment cell	Melanoblastoma, including melanoma and malignant melanoma.
Rhabdomyoblast	Striated muscle	Rhabdomyoblastoma, including rhabdomyoma and rhabdomyosarcoma.

The tables and diagrams are self-explanatory but a few elucidatory notes are appended.

NEOPLASTIC DISEASE 1940–44 INCLUSIVE

TABLE II (A)

BREED INCIDENCE

Breed	*R.I.R.*	*W.L.*	*Br.L.*	*Bl.L.*	*W.W.*	*B.R.*	*L.S.*	*X.*	*Others*	*Total*
Number examined ...	813	398	108	91	126	98	110	60	259	2,063
Number with Neoplastic disease ...	144	78	26	16	13	38	16	12	43	386

From the above, the value of $\chi^2 = 36{\cdot}2$ and with n. = 8 then p. is less than 0·001.

The incidence of Neoplastic disease in the Buff Rock breed is therefore significantly high.

Table II (A) has been arranged so that the Chi-squared test as outlined by Mather (1943) can be employed as a test for significance. It will be seen that out of a total of 2,063 fowls examined, neoplastic disease in some form or other occurred in 386 (18·7 per cent.). This incidence agrees closely with the observations of the New Jersey State Agricultural Experimental Station (1924). Of the nine breeds examined, the Buff Rock and White Wyandotte are prominent in that the former shows a high and the latter a low incidence as compared with the average for all breeds (18·7 per cent.). A statistical check on this apparent feature is therefore desirable.

In the case of the Buff Rock breed, the individual χ^2 (obtained by dividing the difference between the observed number of cases of neoplastic disease and the expected number, squared, by the expected number) is sufficiently high to warrant further investigation. The sum of these values for all breeds is 29·49 which gives a χ^2 of 36·2. To check these results, the contribution to χ^2 by the numbers in each breed without neoplastic disease is computed, and the sum of these contributions, added to the sum of the contributions already obtained, gives a result of 36·06, which agrees closely with the first result.

If Fisher's tables are consulted, it is found that with " n " (the number of degrees of freedom, *i.e.*, in this case the number of different breeds minus one) equal to 8, then the probability that the incidence figures in the Buff Rock breed are high simply by chance is about 1 : 1,000. The conclusion is that they are highly significant.

The next highest contribution to χ^2 is given by White Wyandottes, and in order to test the significance of the apparently low incidence of neoplastic disease in this breed, a 2×2 contingency table has been constructed (Table II (B)) comparing this breed with the remaining breeds but excluding Buff Rocks. This gives a χ^2 of 3·762, and with n = 1, p. lies between 0·1 and 0·05.

To summarise the findings: the greatest deviations from the average incidence is shown by the Buff Rock and White Wyandotte

TABLE II (B)

SIGNIFICANCE TEST FOR WHITE WYANDOTTES

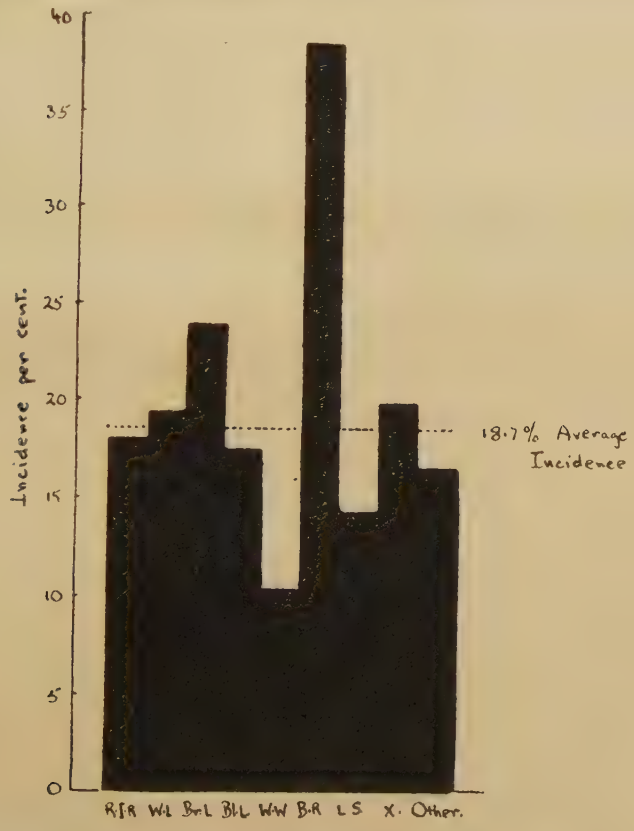
W.W.	(*a*) Number examined 126	(*b*) Number with N.D. 13	$n_1 = 139$
Not W.W. *Less B.R.*	(*c*) Number examined 1,839	(*d*) Number with N.D. 335	$n_2 = 2,174$
	n_3 1,965	n_4 348	$N = 2,313$

The contingency $\chi^2 = \dfrac{(ad - bc)^2 N}{n_1 x\ n_2 x\ n_3 x\ n_4}$

Applying this formula to the above table we get: $\chi^2 = 3{\cdot}762$. With n. = 1, p. lies between 0·1 and 0·05 and the figures for W.W. are therefore significant.

FIGURE I.

SHOWING INCIDENCE OF TUMOURS IN VARIOUS BREEDS OF THE DOMESTIC FOWL.



(The horizontal interrupted line represents the average relative incidence for all the above breeds. The figures for White Wyandotte and Buff Rock are statistically significant.)

breeds. Of the former, nearly 39 per cent. were affected, as compared with a little over 10 per cent. of the latter. None of the other breeds showed statistically significant deviations from the average incidence of 18·7 per cent. Fig. 1 shows in diagrammatic form the incidence in the respective breeds of fowl examined. Table III (A)

TABLE III (A)

SEASONAL INCIDENCE 1940–44 INCLUSIVE

	Jan.	*Feb.*	*Mar.*	*Apr.*	*May*	*June*	*July*	*Aug.*	*Sept.*	*Oct.*	*Nov.*	*Dec.*	*Total*
No. examined	126	97	152	164	176	235	240	206	218	164	192	93	2,063
No. with Neoplastic disease	32	16	47	35	31	43	27	31	33	37	30	24	386

The value of χ^2 as calculated from the above = 37·6 and with n. = 11, *p. i-less than* 0·001.

gives the result of the χ^2 test on the varying incidence of neoplastic disease from month to month over the five-year period. A cursory examination of the figures reveals a high incidence in March and a low one in July, but the annual figures indicate this variation to be constant each year. The calculated result for χ^2 is 37·6, and with n = 11, p. (in Fisher's tables) is less than 0·001. As an additional check, a 2×2 contingency table (Table III (B)) was constructed, comparing summer with winter months, and again p. is found to lie between 0·01 and 0·001.

TABLE III (B)

SIGNIFICANCE TEST FOR INCIDENCE IN SUMMER AND WINTER MONTHS

(*a*) Number examined 1,239	(*b*) Number with N.D. 200	$n_1 = 1,439$
(*c*) Number examined 824	(*d*) Number with N.D. 186	$n_2 = 1,010$
$n_3 = 2,063$	$n_4 = 386$	$N = 2,449$

$\chi^2 = 9·087$

With n. = 1, *p. lies between* 0·01 *and* 0·001.

The difference in incidence between summer and winter is therefore significant and as Table III (A) shows, March and July are the critical months.

It is probable, therefore, that there is a significant seasonal variation of neoplastic disease in the fowl which may be due to increased endocrine activity in the spring causing a flare-up of latent pre-neoplastic cells. The possibility that the figures from month to month might be misleading has not been overlooked. If, for example,

there is a dearth of other diseases in the spring months, the figures for neoplasms, assuming an even distribution throughout the year, would show an apparent increase. Similarly in summer there might be more general disease, making neoplastic disease appear to have a low incidence. This argument could be strengthened by examination of the monthly figures, for it will be seen that the total number of autopsies in the five successive spring months (March) is 153, while the figure for the five summer months (July) is 240. According to this argument, however, and postulating that neoplastic disease is fairly evenly distributed throughout the year, it might be assumed that the highest figure for that disease would appear in December, during which month only 93 autopsies were performed over the five years, whereas in fact it occurs in March. Thus it seems reasonable to conclude there is a seasonal variation.

In further support of these observations, the findings of other workers may be cited. Schaaf (1936) found that over a period of three years the incidence of spontaneous leucosis in fowls had its maximum in March and its minimum from June to October. His explanation was that spring-hatched birds reached the critical age for leukaemia in the following November to May (*i.e.*, 9 to 14 months). While this may be correct in his particular observations, other workers such as Engelbreth-Holm (1942) and Peacock (1935) have reported a similar seasonal variation in the percentage successes in transmission passages of leukaemia and experimentally induced sarcomata. Furthermore, Lambin and Gérard (1934) have observed a seasonal variation in the incidence of leukaemia in man, maximum figures tending to occur in the winter and spring months.

Table V shows the distribution of the various main types of neoplastic condition in the breeds of fowl examined. The figures are based on a four-year survey only but it will be seen by comparison with Fig. 1 that the total incidence is not significantly different. Carcinomata and lymphoblastomata were most common, each accounting for 25·5 per cent. of cases ; " fowl paralysis tumour," or visceral lymphomatosis came next at 18·6 per cent., followed by true leukaemic conditions (mainly erythro- and myeloid leukaemia). The incidence of sarcomata was 6·3 per cent. and plain muscle tumours nearly 4 per cent. The remaining types varied from 3 to 0·3 per cent. The only significant breed difference was the absence of leukaemia from the White Wyandottes and Light Sussex. The failure to find fowl paralysis tumours and aleukaemic lymphocytomata in " other breeds " obviously has no significance.

If lymphoblastoma *per se* and visceral lymphomatosis are grouped together, tumours with lymphoid cells as cell type account for just over 44 per cent. of all types. This finding agrees closely with that of Jackson (1936), who recorded 93 lymphoid tumours in a collection of 203 : incidentally, the incidence of lymphoid tumours in fowl paralysis was 25·6 per cent. Pappenheimer, Dunn and Cone (1926) found 10 per cent. of their cases to have tumours.

TABLE IV (A)

Visceral lymphomatosis	(*a*) Intestines involved 8	(*b*) Intestines not involved 48	$n_1 = 56$
Lymphocytoma	(*c*) Intestines involved 39	(*d*) Intestines not involved 38	$n_2 = 77$
	$n_3 = 47$	$n_4 = 86$	$N = 133$

The contingency $\chi^2 = 14{\cdot}77$. With n. = 1 p. = $<0{\cdot}001$

TABLE IV (B)

Visceral lymphomatosis	(*a*) Liver involved 11	(*b*) Liver not involved 45	$n_1 = 56$
Lymphocytoma	(*c*) Liver involved 43	(*d*) Liver not involved 34	$n_2 = 77$
	$n_3 = 354$	$n_4 = 79$	$N = 133$
	$\chi^2 = 8{\cdot}219$		

With n = 1 p. is between 0·01 and 0·001

In view of the present uncertainty regarding the relationship between lymphocytoma and fowl paralysis tumour (visceral lymphomatosis), it was thought that a comparison of the organ distribution of the two conditions might throw some light on the problem. In 56 cases of lymphoid tumours associated with fowl paralysis, the intestines were involved in eight and the liver in 11. Conversely, in 77 cases of lymphocytoma with no detectable nerve lesions, the intestines were involved in 39 and the liver in 43. Applying the 2×2 contingency test to these figures it is found that in Table IV (A) the contingency χ^2 is 14·77, and with n = 1, p. is less than 0·001, while in IV (B) $\chi^2 = 8{\cdot}219$, and with n = 1, p. lies between 0·01 and 0·001. Thus it appears that in lymphocytoma the intestines and liver are involved in a significantly greater proportion of cases than in visceral lymphomatosis (fowl paralysis). This suggests that there may be an aetiological difference between the two conditions.

TABLE V

A.—PERCENTAGE INCIDENCE OF DEATHS DUE TO NEOPLASIA

B.—SHOWS THE RELATIVE INCIDENCE OF NEOPLASTIC CONDITIONS IN INDIVIDUAL BREEDS OF THE DOMESTIC FOWL, AND IN FOWLS GENERALLY

	A			B																						
				Fowl Paralysis		*Aleukaemic Lymphocytoma*		*Leukaemia*		*Aleukaemic Myelocytoma*		*Carcinoma*		*Sarcoma*		*Leiomyoma*		*Endothelioma*		*Glioma*		*Osteogenic Sarcoma*		*Embryonal Nephroma*	
Breed	*Examined*	*Tumours*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*
Rhode Island Red	653	108	16·5	23	21·2	22	20·3	17	15·7	3	·2·7	27	25·0	5	4·6	3	2·7	5	4·6	2	1·8	0	0·0	1	0·9
White Leghorn... ...	326	61	19·0	10	16·4	21	34·4	4	6·5	2	3·2	15	24·5	3	4·9	2	3·2	0	0·0	1	1·6	0	0·0	3	4·9
Brown Leghorn... ...	90	22	24·4	3	13·6	6	27·2	4	18·1	1	4·5	6	27·2	2	9·0	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0
Black Leghorn	54	11	20·3	2	18·1	3	27·2	4	36·3	0	0·0	2	18·1	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0
White Wyandotte ...	108	11	10·1	3	27·2	2	18·1	0	0·0	0	0·0	3	27·2	1	9·0	2	18·1	0	0·0	0	0·0	0	0·0	0	0·0
Buff Rock ...	87	31	36·7	3	9·6	10	32·2	1	3·2	1	3·2	7	22·5	4	12·9	3	9·6	0	0·0	0	0·0	1	3·2	1	3·2
Light Sussex ...	96	13	12·5	3	23·0	6	46·1	0	0·0	0	0·0	3	23·0	0	0·0	0	0·0	0	0·0	1	7·6	0	0·0	0	0·0
Cross Bred ...	246	39	15·8	9	23·0	7	17·9	3	7·6	2	5·1	11	28·2	3	7·6	2	5·1	2	7·6	0	0·0	0	0·0	0	0·0
Other breeds ...	32	5	15·6	0	0·0	0	0·0	1	20·0	0	0·0	3	60·0	1	20·0	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0
Total	1,692	301	17·7	56	18·6	77	25·5	34	11·2	9	3·0	77	25·5	19	6·3	12	3·9	7	2·6	4	1·3	1	0·3	5	1·6

SUMMARY

During a five-year investigation of spontaneous neoplastic disease in the domestic fowl, 2,063 birds were submitted to detailed examination. In 386 cases of neoplastic disease encountered, with an incidence of 18·7 per cent., 28 distinct types of neoplasia were observed.

Neoplasms were most common in the Buff Rock breed (38·8 per cent.), and least in White Wyandottes (10·3 per cent.). The breed incidence for the various types of neoplastic disease was also investigated, and the only significant difference was the absence of leukaemia in the White Wyandotte and Light Sussex breeds. The seasonal variation showed a maximum incidence in the spring months, particularly March, which was tentatively attributed to the stimulation of latent pre-neoplastic cells by sex hormones.

A comparison of tumour distribution in visceral lymphomatosis (fowl paralysis) and lymphocytoma showed that in the latter the liver and intestines were involved more frequently than in fowl paralysis.

A brief summary of the literature dealing with the incidence of neoplastic disease is given and the problems associated with the classification of avian neoplastic disease are discussed.

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REFERENCES

Babic, I. (1931). *Veterinarski Archiv.*, **1,** 158. (Cited by Goss.)
Bullis, K. L., and Olson, C. (1943). *Amer. J. vet. Res.*, **4,** 382.
Bürger, M. (1914). *Z. f. Krebsforsch.*, **14,** 526.
Butterfield, E. E. (1905). *Folia Haematol.*, **2,** 649.
Caparini, U. (1896). *Clin. Vet.*, **19,** 433. (Cited by Engelbreth-Holm.)
Curtis, M. R. (1915). *J. agric. Res.*, **5,** 397.
Dudley, F. J., Dobson, N., and Gordon, R. F. (1941). *Vet. Rec.*, **53,** 323.
Ehrenreich, M. (1907). *Med. klin. Jahrg.*, **3,** 614.
Ellermann, V. E., and Bang, O. (1908). *Z. Bakt.*, **44,** 595.
Engelbreth-Holm. (1942). *Leukaemia in Animals.* Edinburgh: Oliver & Boyd.
Fölger, A. E. (1917). *Ergebn. allg. Path. path. Anat.*, **18,** 372.
Foulds, L. (1934). *Imp. Canc. Res. Fund. 11th Scientific Rept.* (*Supplement*); (1940). *Amer. J. Canc.*, **39,** 1.
Furth, J. (1931). *Arch. Path.*, **12,** 1.
Goss, L. J. (1940). *Cornell Vet.*, **30,** 75.
Hirschfeld, H., and Jacoby, M. (1909). *Berlin klin. Wschr.*, **4**; (1912). *Zeitschr. klin. Med.*, **75.**
Jackson, C. (1936). *Onderstepoort J.*, **6.**
Jordan, H. E. (1936). *Amer. J. Anat.*, **59,** 249.
Kerr, W. R. (1928). *Vet. J.*, **84,** 584.
Klee. (1917). (Cited by Folger, A. F.)
Kon, J. (1907). *Virchow's Archiv. path. Anat.*, **190,** 338.

Lambin, P., and Gérard, M. J. (1934). "*Sang*," **8**, 730.
Lentz, J. W. (1922). *Poultry Sci.*, **2**, 33.
Malke, E. (1930). *Z. f. Krebsforsch.*, **31**, 47.
Mallory, F. B. (1914). *The Principles of Pathologic Histology.* Philadelphia: W. B. Saunders Co.
Mather, K. (1943). *Statistical Analysis in Biology.*
Matheson, D. C. (1927). *11th World's Poultry Congress*, p. 270.
Matheson, D. C., and Wilson, J. E. (1933). *World's Poultry Congress.* No. 65.
—— *N.J. State Agric. exp. Sta.* (1924).
—— (1929–30). *Rept.* New York State Vet. College, Cornell Univ., Ithaca.
Pappenheimer, A. M., Dunn, L. C., and Cone, V. (1926). *Storrs Agric. exp. Sta. Connecticut. Bull.* 143.
Peacock, P. R. (1935). *Amer. J. Cancer.*, **25**, 49.
Roloff, F. (1868). *Mag. ges. tierheilk.*, **34**, 190.
Rous, P. (1910). *J. exp. Med.*, **12**, 696.
Schaaf, J. (1936). *Z. Infekt. Haustiere.*, **49**, 214.
Schmeisser, H. C. (1916). *John Hopkins Hosp. Rep., Baltimore*, **17**, 551.
Tyzzer, E. E., and Ordway, T. (1909). *J. med. Res.*, **21**, 459.
Warthin, A. S. (1907). *J. infect., Dis.*, **4**, 338.

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